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$1,25(OH)_2D_3$ and its synthetic analog EB1089 induce characteristic features of apoptosis in MCF-7 cells *in vitro*. To determine whether vitamin $D_3$ compounds could mediate apoptosis of breast tumors *in vivo*, we treated nude mice carrying MCF-7 xenografts with the vitamin $D_3$ analog EB1089 for up to five weeks. The volume of tumors from mice treated with 45 pmol/ day EB1089 was four-fold lower than that of tumors from vehicle treated control mice after five weeks. The reduced growth of tumors from EB1089 treated mice was associated with characteristic apoptotic morphology. After five weeks of treatment with EB1089, MCF-7 tumors exhibited a six-fold increase in DNA fragmentation and a two-fold reduction in proliferation relative to control tumors. EB1089 treatment did not alter the growth of xenografts derived from a vitamin $D_3$ resistant variant of MCF-7 cells ($MCF\text{-}7^{D3Res}$ cells), which display resistance to EB1089 *in vitro*, indicating that resistance to EB1089 is maintained *in vivo*. Failure of current endocrine therapies for breast cancer has been attributed to the emergence of hormone independent cells, suggesting that agents like EB1089 which induce apoptosis in both ER+ (estrogen receptor) and ER- cells represent promising adjuncts to existing therapies for breast cancer.

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Kathryn E. VanWinden 6/25/98

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## INTRODUCTION

Despite decades of progress in diagnosis, research, and treatment, breast cancer remains the leading cause of cancer death in women. Anti-estrogen treatment represents the most effective available endocrine therapy, but is limited to the one-third of patients with breast tumors that are estrogen dependent at the time of diagnosis (1). Treatment with anti-estrogens in these patients is often followed by the development of a hormone-resistant phenotype (2). It is clear that effective breast cancer therapies must target both estrogen dependent and independent cells to minimize the development of hormone independent tumors with increased metastatic potential.

1,25 dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), the biologically active form of vitamin $D_3$ (cholecalciferol), is a potent negative growth regulator of both estrogen dependent and independent breast cancer cells *in vitro* (3,4,5,6,7). The vitamin $D_3$ receptor, like the estrogen receptor, is a member of the steroid/ thyroid/ retinoic acid family of nuclear receptors. While the estrogen receptor is present in only two thirds of breast tumors, the vitamin $D_3$ receptor is present in over eighty percent of tumors and does not necessarily colocalize with the estrogen receptor (4,8). Vitamin $D_3$ based therapeutics thus offer promise as either adjunctive agents for estrogen-dependent tumors or as alternative agents for estrogen-independent tumors.

Although it is clear that vitamin $D_3$ compounds inhibit growth of both estrogen receptor positive and estrogen receptor negative breast cancer cells (3,4,5,6,7), the precise mechanism of its effects is unclear. Our lab has characterized the effects of $1,25(OH)_2D_3$ on estrogen receptor positive (ER+) MCF-7 cells and estrogen receptor negative (ER-) SUM 159 cells *in vitro*. Both ER+ and ER- cells are growth inhibited by $1,25(OH)_2D_3$ and its bioactive analogs (9,10,11). We initially demonstrated that $1,25(OH)_2D_3$ induces characteristic features of apoptosis, such as chromatin condensation, nuclear matrix degradation and DNA fragmentation in MCF-7 cells *in vitro* (12,13). Subsequently, we and others reported that apoptosis in breast cancer cells treated with $1,25(OH)_2D_3$ or its synthetic analogs is associated with up-regulation of proteins linked to apoptosis in the mammary gland (such as clusterin and cathepsin B) and down regulation of bcl-2, an anti-apoptotic protein (6,14,15,16). In addition, we demonstrated that growth of MCF-7 xenografts in nude mice was significantly inhibited (40%) in animals treated with the vitamin $D_3$ analog, EB1089 in preliminary studies. Similarly, growth of the MX-1 breast tumor, which is ER-, in nude mice is inhibited 60% after 26 day administration of the 22-oxa analog of $1,25(OH)_2D_3$ (9). A further decrease in tumor weight was observed when the vitamin $D_3$ analog and adriamycin were co-administered. These data suggest that vitamin $D_3$ compounds inhibit growth of breast cancer cells independently of their ER status, and that additive effects of vitamin $D_3$ compounds and other antineoplastic agents can be demonstrated.

The significance of the finding that vitamin D compounds induce apoptosis in breast cancer cells *in vitro* is emphasized by the recognition that many chemotherapeutic agents induce tumor regression via activation of apoptosis (17). For example, apoptosis is induced in estrogen dependent breast tumors during treatment with anti-estrogens such as tamoxifen (12,18). Failure of current anti-hormonal therapies such as tamoxifen is commonly associated with emergence of hormone independent cells (19). Although such estrogen independent cells do not undergo apoptosis following anti-estrogen treatment, these cells retain the ability to undergo apoptosis in response to other agents. Agents which induce apoptosis in estrogen independent cells represent important adjuncts to existing anti-hormonal therapies for breast cancer. As discussed above, estrogen independent cells, such as the BT-20 and SUM159 lines, retain sensitivity to vitamin $D_3$ compounds. Thus, a distinct therapeutic advantage might be achieved by combining therapies that activate vitamin $D_3$ dependent pathways with those that disrupt estrogen dependent signalling. We (12) and others (20,21) have indeed shown complementary effects of vitamin $D_3$ and anti-estrogens in inhibition of breast cancer cell growth *in vitro*. My studies are directed towards demonstrating that vitamin $D_3$ compounds and anti-estrogens exert similar complementary effects on breast tumor regression *in vivo*.

The studies described here examined whether EB1089 could modulate growth of human breast tumors using a nude mice MCF-7 xenograft model, and to determine whether the anti-tumor effects of EB1089 in human breast tumors involve activation of apoptosis. My data demonstrate that EB1089 significantly reduces growth of established human breast tumors by enhancing apoptosis and reducing proliferation of tumor epithelial cells. These data emphasize the potential effectiveness of vitamin $D_3$ based therapeutics for induction of apoptosis in human breast cancer.

## SPECIFIC AIMS & EXPERIMENTAL APPROACHES

I prevoiusly established the dose of EB1089 which causes tumor regression and is tolerated by the mice. I selected the "pure" anti-estrogen ICI 182,780 (generously supplied by Dr. Alan Wakeling) since we have shown that this compound acts cooperatively with EB1089 *in vitro*, and Dr. Wakeling has demonstrated its effectiveness in mediating MCF-7 tumor regression in nude mice (19). These compounds and the nude mouse MCF-7 xenograft animal model will be used for the three specific aims listed below.

**1. To compare tumor regression induced by vitamin $D_3$ compounds and anti-estrogens using MCF-7 cell xenografts grown in nude mice.**
**2. To determine the effects of EB1089 and ICI 182,780 on growth of tumors derived from the vitamin $D_3$ resistant MCF-7 cells.**
**3. To demonstrate that vitamin $D_3$ analogs and anti-estrogens target distinct cells within a mixed tumor.**

The portions of *Specific Aims 1 & 2* studying MCF-7 and MCF-$7^{D3Res}$ xenograft regression using the vitamin $D_3$ analog EB1089 have been completed. The portions of *Specific Aims 1 & 2* studying MCF-7 and MCF-$7^{D3Res}$ xenograft regression using the antiestrogen ICI 182,780 have not yet been completed since the compound was not available at the time the EB1089 studies were performed. The portions requiring ICI 182,780 will be underway shortly. None of the proposed studies in *Specific Aim 3* have been completed at this time. Experimental procedures will be detailed under each *Specific Aim.*

**Specific Aim 1. To compare tumor regression induced by vitamin $D_3$ compounds and anti-estrogens using MCF-7 cell xenografts grown in nude mice.**

**a. Nude mouse tumor regression system.** MCF-7 human breast cancer cells (obtained from ATCC) were cultured in αMEM containing 5% fetal bovine serum (Gibco BRL). Cells were grown in T-150 flasks and yielded 5-10 x $10^6$ cells per flask depending on confluence. For inoculation into nude mice, cells were washed with PBS, trypsinized, resuspended in αMEM and pooled. After centrifugation, cells were resuspended in Matrigel (Collaborative Biomedical)/ αMEM (4:1).

Three series of studies were conducted to examine the effects of EB1089 on growth and apoptosis of MCF-7 xenografts. In all studies, ovariectomized Ncr *nu/nu* mice (Taconic, Germantown, NY) were implanted sc with 17β-estradiol sustained release pellets (Innovative Research, Sarasota, FL). The mice were fed a low calcium (0.1%) purified rodent chow (Purina Test Diets, Richmond, IN) for the duration of the study to minimize calcemic effects of vitamin $D_3$ analog treatment. Mice were inoculated sc with approximately 5 x $10^6$ MCF-7 cells suspended in 0.3 ml Matrigel/ αMEM. Tumor take rate ranged from 95-100%. Tumor volumes were monitored weekly by caliper measurement of the length, width, and height, and the tumor volume was calculated using the formula for a semi-ellipsoid ($4/3\pi r^3/2$). After three weeks, mice bearing tumors with volumes averaging approximately 200 $mm^3$ were randomized for treatment. Because of the variations in tumor take and initial tumor growth, as well as the removal of mice for analysis at various time points, the number of mice at each time point varied from experiment to experiment. The number of mice analysed are reported either in the text or in the figure legends.

In the first series of studies, MCF-7 tumor bearing mice were treated with EB1089 at a dose of 60 pmol per day. This dose was chosen based on preliminary studies in non-tumor bearing balb/c mice, which indicated that doses up to 90 pmol/ day could be tolerated with little weight loss or elevation of serum calcium when animals were fed the low calcium diet (data not shown). EB1089 was suspended in 80% propylene glycol/ 20% PBS and administered daily via sc injection. Control mice received daily injections of the vehicle alone. In a third group of tumor bearing mice, which served as a positive control, estradiol pellets were removed to induce apoptotic tumor regression as reported by Kyprianou et al (26). Tumor volumes were monitored weekly, and mice were sacrificed after two to five weeks of treatment.

In the second series of studies, the dose of EB1089 was lowered to 45pmol/ day, since mice given 60 pmol of EB1089 per day experienced weight loss and hypercalcemia. In this experiment, mice bearing MCF-7 tumors were randomized into control (n=14), EB1089 treated (n=16) and estradiol withdrawal (n=3) groups.

To investigate whether EB1089 could be administered via sustained release pellets similar to those used for estrogen supplementation, a preliminary study was conducted with custom made pellets (Innovative Research) designed to continuously release 30, 45, or 60 pmol of EB1089 per day for five weeks. MCF-7 xenografts were established from MCF-7 cells and EB1089 or placebo pellets were implanted ip under sodium pentobarbital anesthetic three weeks after inoculation. Tumor volumes were measured and treatment was terminated after five weeks. Due to the small numbers of mice in each group (EB1089 pellets, n=3 for 30pmol, n=1 each for 45 or 60pmol; Control, n=4), the data for all three doses were combined in the analysis.

A study using ICI 182,780 (10μg/kg BW, injected s.c.), a dose which has been shown to induce regression of MCF-7 xenografts in nude mice (19), will be conducted shortly to complete this portion of *Specific Aim 1.*

**b. Tumor analysis.** Upon termination of treatment, tumors were excised and weighed, and portions of each were frozen for homogenization and processing for SDS-PAGE and western blotting. Portions of each tumor were also fixed for light microscopy and immunofluoresence. Tumors were embedded in paraffin, sectioned at 5 μM and stained with hematoxylin (Gill's formulation #3, Fisher) and Eosin Y (Sigma). The epithelial nature of the tumors was verified by immunostaining with antibodies directed against epithelial specific antigen and cytokeratin 18. Mitotic index and apoptotic index were assessed by quantitative morphometric analysis of proliferating cell nuclear antigen (PCNA) expression and *in situ* terminal transferase mediated fluorescein dUTP nick end labeling (TUNEL), two established markers of proliferation and apoptosis. For PCNA localization, formalin fixed, paraffin embedded sections were incubated 30 minutes with a mouse monoclonal anti-PCNA (Nova Castra) at a 1:100 dilution in 1% BSA/ PBS. A biotin-conjugated antibody to mouse IgG (Vector) was applied at a 1:200 dilution for 30 minutes in 1% BSA/ PBS. The ABC technique was used (avidin and biotinylated horseradish peroxidase complex, Vector) followed by diaminobenzidine (Sigma) to localize peroxidase in the sections, and the sections were counterstained with hematoxylin (Harris modified, Fisher). DNA fragmentation was assessed by TUNEL using the commercially available assay according to manufacturer's directions (Boehringer Mannheim). In these sections, nuclei were counterstained with Hoechst 33258 dye (Sigma).

PCNA expression and TUNEL were quantitated by viewing and photographing two random fields of each tissue section on a Nikon Optiphot-2 microscope and Nikon Microflex UFX-IIA photomicrographic attachment using a 40x objective. The photographs were scanned and analyzed with the USTHSCSA *Image Tool* program and the percentage of cells staining positively for PCNA or TUNEL awas calculated. For both TUNEL and PCNA, two-six fields of view (containing at least 250 cells) were quantitated on each section, with four to eight samples evaluated for each treatment per time point.

**c. Calcium homeostasis and organ histology.** Body weights were monitored weekly. At the termination of treatment (2-5 weeks), mice were anesthetized with sodium pentobarbital and blood was collected by cardiac puncture for serum calcium determination. Serum calcium level was determined using the colorimetric calcium reagent Arsenazo III (Sigma). Target tissues such as intestine, bone, and kidney were processed as described above for tumors, and examined for effects of EB1089 on calcium homeostasis and histology. Uterine size was examined as an indicator of estrogen status to ensure that all mice receive adequate circulating estrogens from the implanted pellets.

**d. Statistical analyses.** Statistical comparisons were performed by Students unpaired t test (for two groups) or one way analysis of variance (ANOVA) for more than two groups. Data are expressed as mean ± standard error, and differences between means were considered significant if p values less than 0.05 were obtained.

**Specific Aim 2. To determine the effects of EB1089 and ICI 182,780 on growth of tumors derived from the vitamin $D_3$ resistant MCF-7 cells.**

**a. Nude mouse tumor regression system.** The vitamin $D_3$ resistant variant of MCF-7 cells (MCF-$7^{D3Res}$ cells) which has been previously described (28), are resistant to the growth inhibitory effects of EB1089 *in vitro*. MCF-$7^{D3Res}$ cells were cultured as described for MCF-7 cells in *Aim 1*. To examine whether EB1089 induced non-specific or indirect effects on tumor growth kinetics, tumors were established from MCF-$7^{D3Res}$ cells. Mice bearing

MCF-$7^{D3Res}$ tumors were randomized into control (n = 8) and 45pmol/ day EB1089 treated (n = 6) groups in one experiment, and into control (n = 3) and estradiol withdrawal (n = 3) groups in another trial. The experimental design for this study was otherwise identical to those described above for studies employing the 60pmol/day dose or 45pmol in MCF-7 xenografts.

A study using ICI 182,780 (10μg/kg BW, injected s.c.) will be conducted shortly to complete this portion of *Specific Aim 2*.

**b. Tumor analysis, c. Calcium homeostasis, d. Statistical analyses.** As described for studies in *Specific Aim 1*.

**Specific Aim 3. To determine whether combination treatment with vitamin $D_3$ analogs and anti-estrogen enhance tumor regression compared to either treatment alone.**

We anticipate that the data from *Aims 1 and 2* will support our hypothesis that EB1089 and ICI 182,780 affect different subsets of cells within the tumor *in vivo*. If so, then combination therapy with both agents should enhance tumor regression compared to treatment with either agent alone. We (12) and others (20,21) have demonstrated complementary effects of vitamin $D_3$ compounds and anti-estrogens *in vitro*, and my next goal will be to extend these findings to the tumor regression model. Tumor bearing mice will be randomized into 4 groups: group 1 will receive no treatment; group 2 will receive ICI 182,780 ; group 3 will receive EB1089; and group 4 will receive both ICI 182,780 and EB1089. Body weights and tumor volumes will be measured weekly and 8 animals per group will be killed each week for 4 weeks. The tumors will be processed for analysis as described for Aims 1 and 2. Since we will monitor each parameter at weekly intervals we will be able to determine the relative efficacy of each treatment over time. Thus, even if final tumor volumes are small, we will be able to determine whether the combination therapy induces more rapid and/or greater tumor regression.

## RESULTS

**Specific Aim 1. To compare tumor regression induced by vitamin $D_3$ compounds and anti-estrogens using MCF-7 cell xenografts grown in nude mice.**

**a. Nude mouse tumor regression system. Effect of 60 pmol EB1089 on MCF-7 tumor growth kinetics.** Tumor growth kinetics in ovariectomized mice treated with 60 pmol EB1089 per day or vehicle, in the presence of estradiol supplementation, are presented in Figure 1. In control mice, average tumor volume increased rapidly over the three week treatment period. In EB1089 treated mice, tumor volume increased during the first week of treatment and then plateaued, with no further increase in tumor volume between weeks two and three. In control mice, the mean change in tumor volume between the first and third weeks was 272.3 ± 113.2 $mm^3$ (n=4), compared to 3.6 ± 15.6 $mm^3$ (n=5) for tumors from EB1089 treated mice ($p<0.05$). Tumor volume after two weeks was significantly lower in the EB1089 group (351.2 ± 80.4 $mm^3$, n=10) than in the control group (631.1 ± 65.9 $mm^3$, n=7). In mice subjected to removal of the estradiol supplementation, tumors regressed rapidly, becoming undetectable within three weeks, demonstrating the estrogen dependence of the MCF-7 xenografts.

**Effect of 45 pmol EB1089 on growth of MCF-7 tumors.** Although significant effects of EB1089 on MCF-7 tumor growth kinetics were observed in our initial studies, the low tolerance of the tumor bearing mice to the 60pmol/day dose precluded definitive conclusions regarding the specificity of this effect. Therefore, a second series of studies using a lower (45 pmol) dose of EB1089 were conducted. Temporal changes in tumor volume for animals bearing MCF-7 tumors and treated with 45 pmol/ day EB1089 or vehicle are shown in Figure 2. This graph shows mean tumor volumes for all animals which completed the five week study protocol (ie, not including tumors which were removed for histological analysis at various times). Consistent with data shown in Figure 1 for the 60pmol/day dose, growth of MCF-7 tumors in mice treated with 45pmol/day EB1089 was slower than that of tumors from vehicle treated control animals from one week on. The mean change in tumor volume between the first and fifth weeks was 366.6 ± 53.6 $mm^3$ (n=6) in control mice, compared to 53.2 ± 56.9 $mm^3$ (n=6) in EB1089 treated mice ($p< 0.01$). Tumor volume after five weeks was significantly ($p<0.01$) lower in the EB1089 treated group (428.6 ± 274.0 $mm^3$, n=6) than in the control group (1716.0 ± 217.7 $mm^3$, n=6). Final tumor weight was significantly ($p<0.01$) lower in EB1089 treated mice (0.43 ± 0.27 g, n=6) than in control mice (1.52 ± 0.19 g, n=6). These data indicate a good correlation between tumor volume assessed by caliper measurement in live mice and actual tumor size measured after sacrifice. The anti-tumor effect of EB1089 persisted over the entire five week experiment. In addition, although not readily evident from the graph (which shows mean tumor volume) two MCF-7 tumors completely regressed in response to EB1089 treatment. Tumors which regressed were monitored for up to two months in the absence of EB1089 treatment, and no re-growth was observed (data not shown). In contrast, spontaneous regression was never observed in tumors from control mice.

**Effect of EB1089 sustained release pellets on growth of MCF-7 tumors.** In a third, preliminary study, we examined whether EB1089 could be administered via sustained release pellets similar to those used for estradiol supplementation. Pellets designed to continuously release 30, 45, or 60 pmol of EB1089 per day, or placebo pellets, were implanted into mice bearing established MCF-7 tumors. Due to the small number of animals (4 control and 5 EB1089 treated mice) used in this trial, data from the three EB1089 treatment groups were pooled for analysis as there were no obvious differences in response to the three doses. As presented in Figure 3, administration of EB1089 via sustained release pellets elicited a similar anti-tumor response as observed in Figures 1 and 2 for 60 and 45pmol sc injections. Tumors from mice implanted with EB1089 pellets grew at a slower rate than tumors from mice implanted with placebo pellets from one week on. The mean change in tumor volume between the first and fifth weeks was 1594.3 ± 418.7 (n=4) in mice bearing placebo pellets, compared to 285.8 ± 164.3 (n=5) in mice bearing EB1089 pellets ($p< 0.01$), including one EB1089 treated tumor which regressed completely. Final tumor volume at five weeks was significantly ($p<0.05$) lower in the EB1089 pellet group (619.7 ± 256.0, n=5) than in the placebo group (2189.6 ± 554.8, n=4). There were no significant differences in body weights (control, 24.6 ± 0.2, n=5; EB1089 pellets, 23.6 ± 1.5, n=4) or serum calcium (control, 9.5 ± 0.2, n=5; EB1089 pellets, 9.6 ± 0.2, n=4) between mice bearing placebo or EB1089 pellets after five weeks.

**b. Tumor analysis. Effect of EB1089 on morphology, apoptosis and mitosis of MCF-7 tumors.** The epithelial nature of MCF-7 xenografts was verified by immunostaining sections of untreated tumors with an antibody directed

against epithelial specific antigen and cytokeratin 18 (Figure 4). Sections from MCF-7 tumors taken from mice treated with 45 or 60 pmol/day EB1089 or vehicle for two-five weeks were stained with hematoxylin and eosin for assessment of general morphology. The histological appearance of tumors from mice treated with 45pmol or 60pmol per day were similar, and representative micrographs from the two week time point are presented in Figure 5. Tumors from vehicle treated mice were primarily composed of tumor epithelial cells, with small amounts of mouse-derived stroma and frequent blood vessels. The majority of epithelial cells in control tumors were quiescent, although mitotic figures were visible in most sections. Tumors from mice treated with 60 pmol/ day EB1089 were composed primarily of quiescent epithelial cells, with few mitotic figures. In many areas, epithelial cells with classic apoptotic morphology (condensed cells with pyknotic nuclei) were frequent. Many EB1089 treated tumors displayed large areas of stroma where deletion of epithelial cells had occurred. The extent of vascularization appeared equivalent in tumors from EB1089 treated and control mice.

To determine whether the changes in MCF-7 tumor morphology induced by EB1089 were associated with changes in apoptotic or mitotic index, we quantitated the extent of DNA fragmentation (assessed as TUNEL positive cells) as a marker of apoptosis and expression of PCNA as a marker of proliferation. Representative sections stained with Hoechst dye to visualize nuclear morphology and processed for TUNEL are presented in Figure 6. Nuclei of epithelial cells in MCF-7 tumors from control mice exhibited normal morphology and were generally negative for DNA fragmentation. In contrast, sections of tumors from EB1089 treated mice exhibited a high prevalence of condensed, irregularly shaped nuclei which were positive for DNA fragmentation. Tumors from mice which had been subjected to estradiol withdrawal also exhibited irregularly shaped, condensed nuclei which were positive for DNA fragmentation. The morphology of tumors from estradiol deprived mice was similar to that of tumors from EB1089 treated mice, although the extent of apoptosis was higher after estradiol deprivation than after EB1089 treatment (Figure 6). This finding is consistent with the more pronounced tumor regression in mice subjected to estradiol withdrawal compared to EB1089 treatment (Figures 1-3). Quantitative analysis indicated an increase in the percentage of TUNEL positive cells in tumors from EB1089 treated mice compared to tumors from control mice at all time points examined (Table 1). After two weeks of treatment, the higher dose of EB1089 (60pmol/day) was associated with a more pronounced increase in apoptotic index than the 45pmol/day dose. After five weeks of treatment, however, both doses of EB1089 were equivalent, with tumors from treated mice exhibiting a six fold increase in the percentage of cells positive for DNA fragmentation compared to control tumors.

Expression of PCNA was examined as a marker of proliferation in MCF-7 tumor sections derived from control and EB1089 treated mice (Figure 7). In control tumors, PCNA expression was detected in the nucleus of approximately half of all epithelial cells. A decrease in PCNA expression was detected in tumors from EB1089 treated mice at all time points examined. Quantitation of PCNA expression after two weeks indicated a 2.5 fold reduction in PCNA staining in EB1089 treated tumors compared to control tumors (Table 1). Treatment with 45 and 60 pmol/day EB1089 were equally effective in down regulation of PCNA expression at both time points. The reduced expression of PCNA in EB1089 treated tumors compared to control tumors was maintained throughout the five weeks of treatment.

***Anticipated results:*** Based on our data derived from MCF-7 cells grown *in vitro* (15), as well as previous work demonstrating up regulation of apoptosis marker genes during mammary regression (30), we expect to detect increases in clusterin, cathepsin B and transglutaminase during breast tumor regression in response to EB1089 *in vivo*. These studies will also indicate whether the induction of apoptosis after anti-estrogen treatment and EB1089 display similar kinetics, and whether these agents induce a common set of proteins during apoptotic regression. Comparison of tumor size, mitotic and apoptotic indices and the steady state levels of apoptotic marker proteins will indicate whether ICI 182,780 and EB1089 induce tumor regression by similar mechanisms.

**c. Calcium homeostasis and organ histology.** In contrast to our preliminary data which indicated that non-tumor bearing nude mice could tolerate doses of EB1089 up to 90 pmol/ day, tumor bearing mice treated with 60 pmol/ day EB1089 experienced weight loss and elevated serum calcium despite being maintained on the low calcium diet (Table 2). As indicated in Table 3, although serum calcium was elevated in mice treated for five weeks with 45 pmol/ day EB1089 compared to control mice, the levels were not as high as in mice receiving 60pmol/day (Table 2). Furthermore, the significant loss of body weight which was evident with the 60 pmol/ day treatment was not observed when EB1089 was given at the 45 pmol/ day dose. Corrected final body weight (mouse body weight

minus tumor weight) was similar for EB1089 treated and control mice. Intestine, bone, and kidney from animals treated for five weeks with 45 pmol/ day EB1089 or vehicle were examined for effects of EB1089 on calcium homeostasis and histology. No differences in morphology between the two treatment groups were observed, and no soft tissue calcifications were apparent.

**Specific Aim 2. To determine the effects of EB1089 and ICI 182,780 on growth of tumors derived from the vitamin $D_3$ resistant MCF-7 cells.**

**a. Nude mouse tumor regression system. Effect of 45 pmol EB1089 on growth of tumors derived from MCF-$7^{D3Res}$ cells.** In contrast to tumors derived from MCF-7 cells, tumors derived from MCF-$7^{D3Res}$ cells failed to respond to EB1089 treatment (Figure 8). There were no significant differences in MCF-$7^{D3Res}$ tumor volumes of control or EB1089 treated mice at any time point throughout the five week experiment. As demonstrated in Figure 8, MCF-$7^{D3Res}$ tumors underwent rapid regression in response to estradiol withdrawal. Thus, despite resistance to the anti-tumor effects of EB1089, tumors derived from the MCF-$7^{D3Res}$ cells have retained the ability to undergo regression in response to estradiol deprivation, which is known to induce apoptosis in MCF-7 tumors (26).

**b. Tumor analysis.** In contrast to the large areas of cellular deletion and increased stroma seen in MCF-7 tumors treated with EB1089, no morphlogical differences were observed between control and EB1089 treated tumors (Figure 9). In comparison to MCF-7 tumors, untreated MCF-$7^{D3Res}$ tumors exhibit a more differentiated looking morphology, with prominent round glandular-like structures throughout (compare Figure 9 to Figure 5).

Tumor sections from vehicle and EB1089 treated tumors were processed for TUNEL to examine relative levels of DNA fragmentation , however no differences were observed between the two treatments (Figure 10). This result is consistent with the lack of effect of EB1089 on MCF-$7^{D3Res}$ tumor growth kinetics.

***Anticipated results:*** The MCF-$7^{D3Res}$ cells were selected for growth in $1,25(OH)_2D_3$ and display cross resistance *in vitro* to vitamin $D_3$ analogs including EB1089 (28). In contrast, the MCF-$7^{D3Res}$ cells are sensitive to anti-estrogens *in vitro* (28), thus we expect to observe comparable regression and induction of apoptotic markers in tumors derived from MCF-$7^{WT}$ and MCF-$7^{D3Res}$ cells following ICI 182,780 treatment. The receptor analysis will indicate whether treatment responsiveness correlates to the presence of the appropriate receptors, and will also provide baseline data for *Aim 3*. These studies should conclusively demonstrate a dissociation between vitamin $D_3$ and anti-estrogen mediated apoptosis in this model of human breast cancer.

**c. Calcium homeostasis.** There were no significant differences in body weight or serum calcium in mice bearing tumors derived from MCF-7 cells compared to those derived from MCF-$7^{D3Res}$ cells, whether treated with EB1089 or not (data not shown).

**Specific Aim 3. To determine whether combination treatment with vitamin $D_3$ analogs and anti-estrogen enhance tumor regression compared to either treatment alone.**

***Anticipated results:*** We predict that our *in vivo* data will confirm our *in vitro* results that vitamin $D_3$ treatment potentiates the effects of anti-estrogens on breast cancer cell growth. Similar cooperative effects of a distinct vitamin $D_3$ analog (22-oxa-$1,25(OH)_2D_3$) and the anti-estrogen tamoxifen have been demonstrated (14), but no assessment of tumor morphology, mitosis or apoptosis was conducted to determine the mechanism of this interaction. Our measurements of proliferation and cell death at each time point will establish whether additive effects of ICI 182,780 and EB1089 are at the level of mitosis or apoptosis or both. The morphology and immunofluorescent analyses will determine whether the combined therapy exerts additive effects on clusterin, transglutaminase and cathepsin B expression.

As discussed above, anti-estrogen treatment is often associated with selection of hormone independent cells which lack functional ER. Mapping of VDR and ER expression in tumors from Aims 2 and 3 will indicate if either EB1089 or ICI 182,780 treatment results in the emergence of resistant cells lacking either receptor. In addition, we will determine whether estrogen independent cells retain VDR (which would suggest sensitivity to vitamin $D_3$),

and whether EB1089 resistant cells retain ER (which would suggest sensitivity to anti-estrogens). Perhaps most importantly, we will be able to determine whether combined therapy prevents the emergence of resistant cells.

## FIGURES

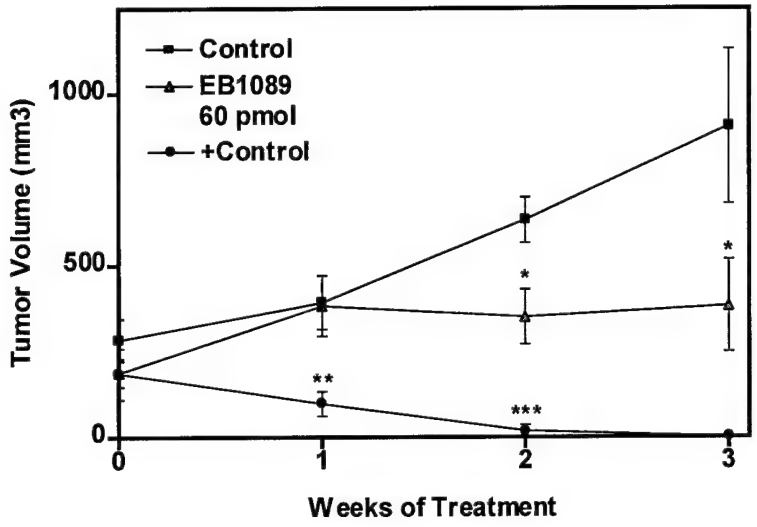


**Figure 1.** Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were given daily sc injections of 60 pmol EB1089 or vehicle beginning three weeks after tumor cell inoculation. In a separate group of mice, estradiol pellets were removed at time zero and no further treatment was given. In all animals, tumor volumes were monitored weekly. Data are expressed as mean ± standard error, control n=4, EB1089 60 pmol n=5, and +control n=3. *p < 0.05; **p< 0.01; ***p<0.001 as assessed by Student's unpaired t test of treatment groups (EB1089 or estradiol withdrawal) compared vehicle. (Data points also significant by ANOVA).

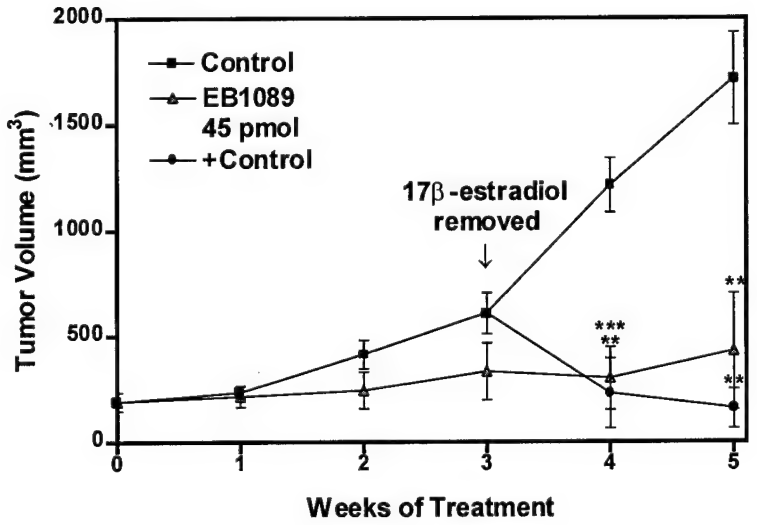


**Figure 2.** Ovariectomized nude mice supplemented with estradiol were inoculated with MCF-7 cells and allowed to grow for three weeks. Mice bearing established tumors were then given daily sc injections of 45 pmol EB1089 or vehicle for five weeks. In a subset of vehicle treated mice, estradiol pellets were removed at week three and no further treatment was given. In all animals, tumor volumes were monitored weekly. Data are expressed as mean ± standard error, control n=6, EB1089 45 pmol n=6, and +control n=2. *p < 0.05; **p< 0.01; ***p<0.001 as assessed by Student's unpaired t test of treatment groups (EB1089 or estradiol withdrawal) compared vehicle. (Data points also significant by ANOVA).

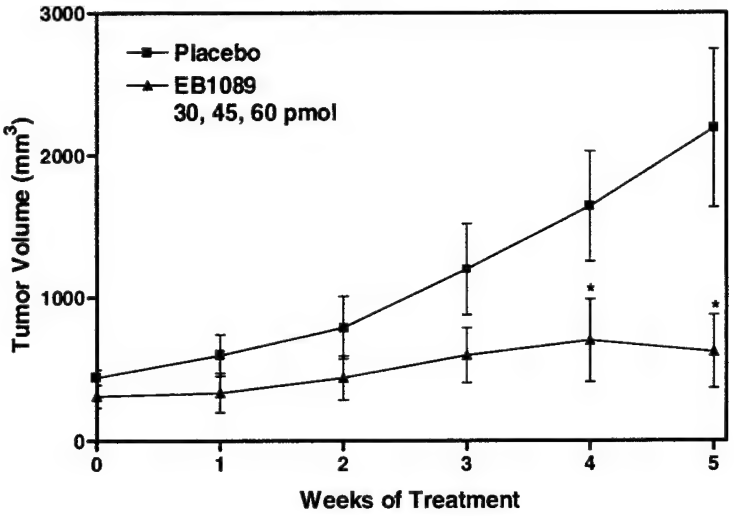


**Figure 3.** Effect of EB1089 Sustained Release Pellets on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were implanted ip with sustained release EB1089 pellets, and tumor volumes were monitored for five weeks. Data are expressed as mean ± standard error of 4 control and 5 EB1089 treated mice. *p < 0.05; **p< 0.01; ***p<0.001.

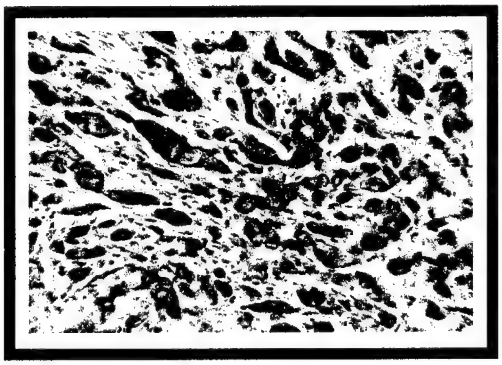

**Figure 4.** Pan-Cytokeratin was localized using the peroxidase method for immunostaining. Positive staining is seen as a brown color.

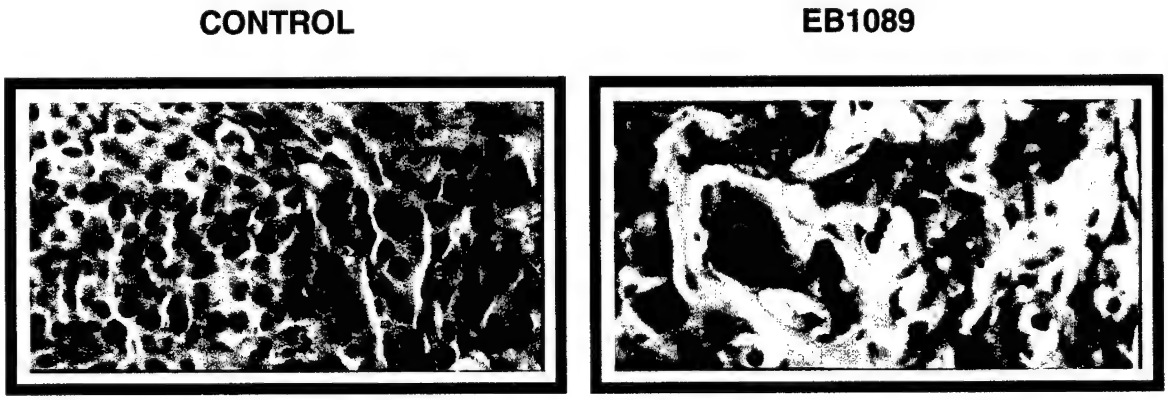


**Figure 5.** Representative tumor sections from mice treated with vehicle (left) or 60 pmol/ day EB1089 (right) for two weeks after staining with hematoxylin and eosin (H & E).

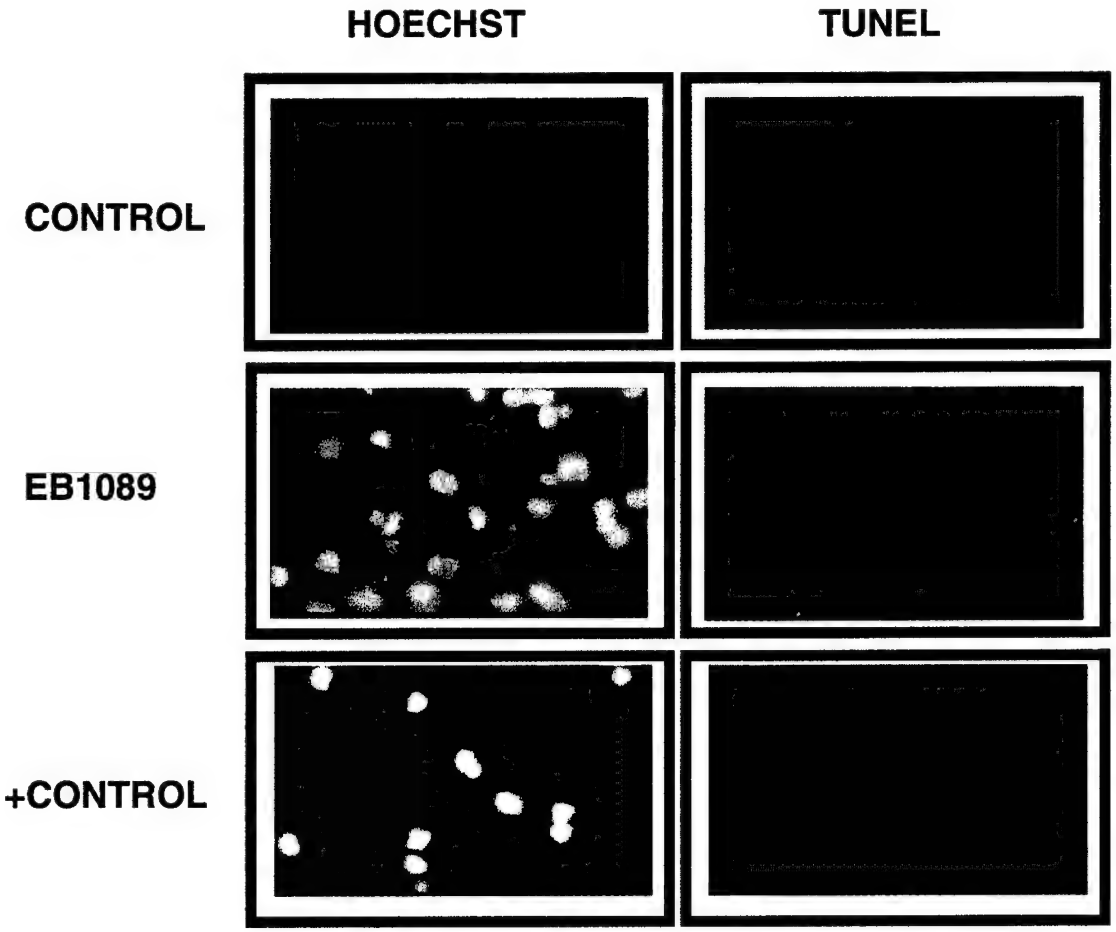


**Figure 6.** MCF-7 tumor sections from mice treated with vehicle (top panels), 60 pmol/ day EB1089 (middle panels) or subjected to removal of estradiol supplementation (bottom panels).

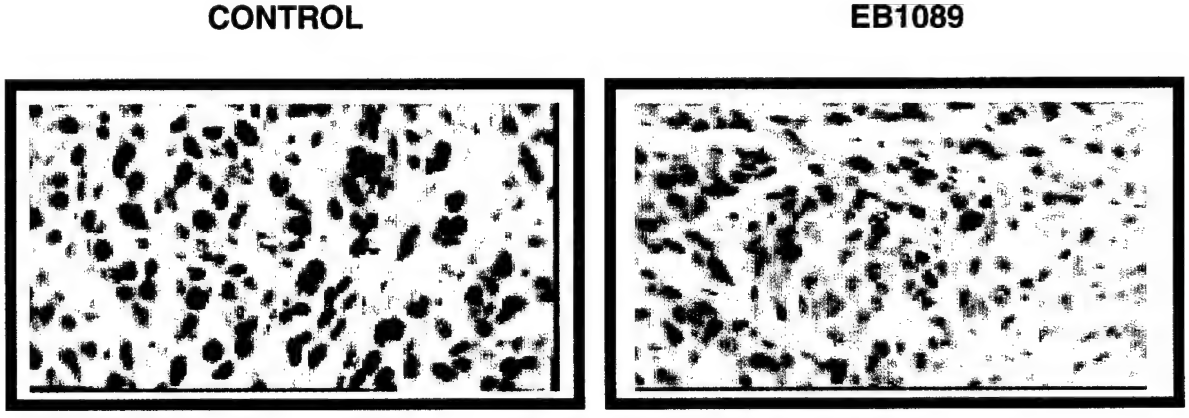


**Figure 7**. Mice were treated with vehicle (left) or 60 pmol/ day EB1089 (right) for two weeks. PCNA expression appears as brown nuclear stain.

| 2 Weeks Treatment: | % PCNA | % TUNEL |
|---|---|---|
| Vehicle | 62.31 ± 3.22 | 7.45 ± 1.37 |
| EB1089 45 or 60 pmol | 24.93 ± 5.24*** | 44.73 ± 5.06*** |
| **4 Weeks Treatment:** | | |
| Vehicle | 65.08 ± 2.22 | 6.11 ± 2.44 |
| EB1089 45 or 60 pmol | 28.80 ± 4.68*** | 53.19 ± 10.30** |
| **5 Weeks Treatment:** | | |
| Vehicle | 59.64 ± 3.76 | 8.38 ± 1.53 |
| EB1089 45 or 60 pmol | 28.00 ± 5.81*** | 61.22 ± 3.27*** |

**Table 1.** Data are expressed as mean ± standard error of at least 500 cells counted in a minimum of two different fields of view . Differences between groups considered significant when p<0.0.5 (*), p<0.01 (**), and p<0.001 (***).

| 2 Weeks of Treatment: | Vehicle | EB1089 |
|---|---|---|
| Serum Calcium (mg/dL) | 8.59 ± 0.21 (n=2) | 12.87 ± 0.05* (n=2) |
| Body Weight (g) | 21.23 ± 0.84 (n=4) | 19.08 ± 1.00 (n=5) |
| **3 Weeks of Treatment:** | **Vehicle** | **EB1089** |
| Serum Calcium (mg/dL) | 9.41 ± 0.08 (n=5) | 11.75 ± 1.23** (n=2) |
| Body Weight (g) | 20.20 ± 0.31 (n=4) | 16.56 ± 1.23* (n=5) |

**Table 2.** Body weights and serum calcium were measured after two or three weeks of treatment with 60 pmol/ day EB1089 or vehicle. Data are expressed as
mean ± standard error. Differences between groups were considered significant when p<0.0.5 (*), p<0.01 (**), and p<0.001 (***).

| 5 Weeks of Treatment: | Vehicle | EB1089 |
|---|---|---|
| Serum Calcium (mg/dL) | 9.70 ± 0.26 (n=9) | 10.88 ± 0.67* (n=6) |
| Body Weight (g) | 23.68 ± 0.39 (n=6) | 22.81 ± 0.52 (n=6) |

**Table 3**. Body weights and serum calcium were measured after five weeks treatment with 45 pmol/ day EB1089 or vehicle. Data are expressed as mean ± standard error . Differences between groups were considered significant when p<0.0.5 (*), p<0.01 (**), and p<0.001 (***).

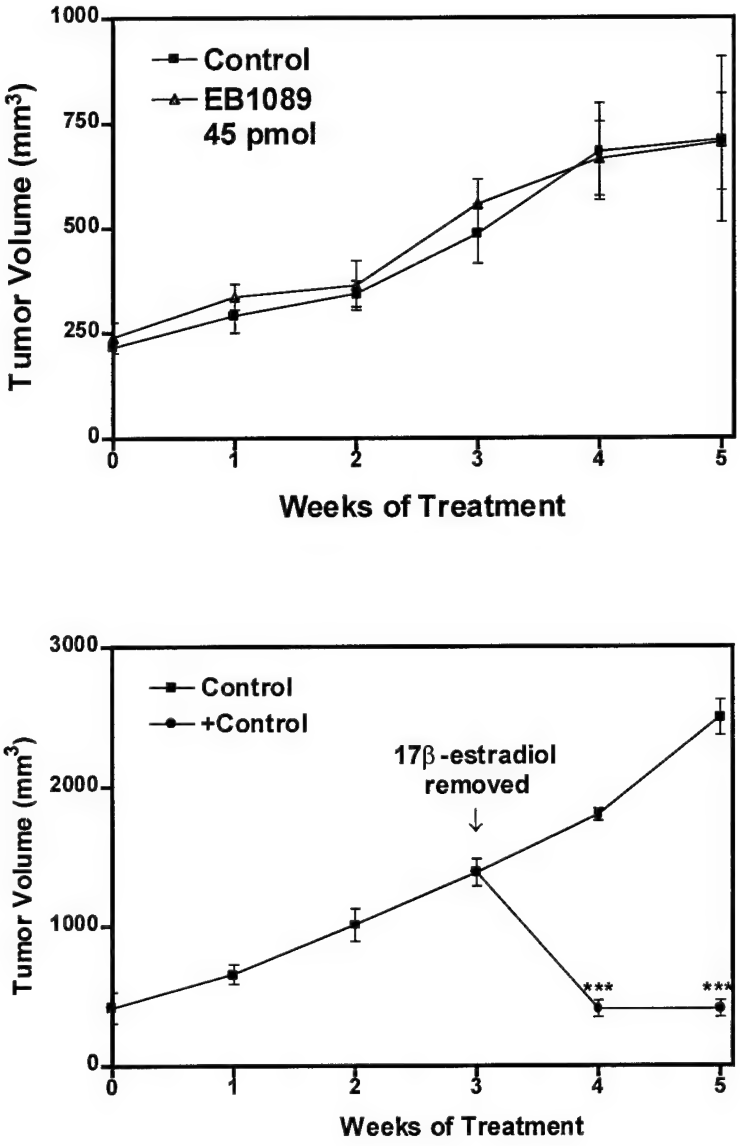


**Figure 8.** *Top.* Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-$7^{D3Res}$ cells were given daily sc injections of 45 pmol EB1089 or vehicle for five weeks. Tumor volumes were monitored weekly. Each point represents mean ± standard error of 8 control mice and 6 EB1089 treated mice. *Bottom.* Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-$7^{D3Res}$ cells were subjected to estradiol withdrawal. In control mice, estradiol pellets were left intact. Tumor volumes were monitored weekly. Each point represents mean ± standard error of 3 control mice and 3 mice subjected to estradiol withdrawal. ***Significantly different, control vs. estradiol withdrawal, $p<0.001$.

**Figure 9.** Representative MCF-7 $^{D3Res}$ tumor sections from mice treated with vehicle (left) or 45 pmol/ day EB1089 (right) for five weeks.

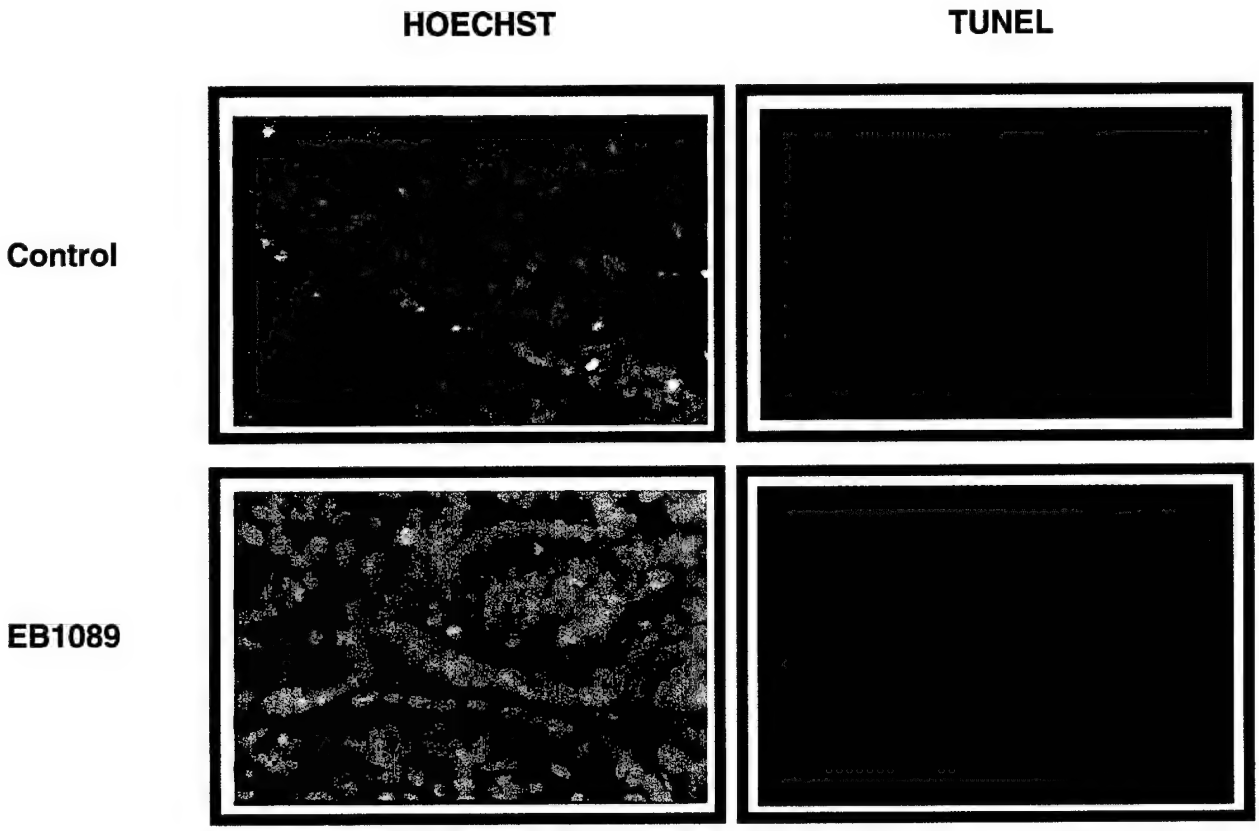


**Figure 10.** MCF-7$^{D3Res}$ tumor sections from mice treated with vehicle (top panels) or 45 pmol/ day EB1089 (bottom panels).

## DISCUSSION

In this series of *in vivo* studies we have demonstrated that the vitamin $D_3$ analog EB1089 significantly reduces the growth of estrogen dependent MCF-7 human breast tumors. In our studies, tumor volumes from nude mice treated with EB1089 by daily sc injection at a dose of 45 pmol/ mouse/ day (approximately 0.8 µg/ kg body weight) were four fold less than those of vehicle treated mice. At this dose of EB1089, serum calcium was minimally elevated and no weight loss was observed. These findings complement those of Colston's group (22-25) who demonstrated that oral administration of EB1089 at doses up to 1µg/ kg body weight daily slowed the growth of established NMU - induced rat mammary tumors without induction of hypercalcemia. In our nude mice studies, treatment with 60 pmol/ mouse/day EB1089 had a more pronounced anti-tumor effect than treatment with 45pmol/day, but the higher dose was associated with hypercalcemia, weight loss and mortality. Preliminary studies utilizing sustained release pellets designed to continuously release EB1089 at doses up to 60 pmol/ day indicated that this mode of administration induced anti-tumor effects similar to those achieved with daily injections. This preliminary data indicated that pellet delivery of EB1089 for five weeks was not associated with hypercalcemia or weight loss.

Histological examination indicated that the decreased size of tumors from EB1089 treated mice, compared to control mice, was associated with a reduction in the epithelial component, and an increase in the stroma (Figure 5). The four fold reduction of tumor volume in mice treated with 45 pmol/ day EB1089 for five weeks (Figure 2) could reflect a decreased rate of cell proliferation, an increased rate of cell death, or both. Our analyses confirmed that EB1089 mediates tumor regression by modulation of both apoptosis and proliferation of tumor epithelial cells.

Quantitative morphometric analysis of DNA fragmentation indicated that tumors from EB1089 treated mice exhibited apoptotic morphology and a six fold increase in the percentage of TUNEL positive cells compared to tumors from control mice. Our studies also demonstrated that MCF-7 tumor regression and DNA fragmentation induced by EB1089 was morphologically similar to tumor regression resulting from estradiol withdrawal, which is known to induce apoptosis in estrogen dependent MCF-7 tumors (26). Our data demonstrating induction of apoptosis in MCF-7 tumors *in vivo* correlate with earlier findings which demonstrated that $1,25(OH)_2D_3$ and its structural analogs induce apoptosis in MCF-7 cells *in vitro* (3,4,12,13,14,15). In addition to induction of apoptosis, EB1089 treated tumors exhibited a significant decrease in proliferation, as measured by PCNA expression, at all time points examined. These data are consistent with flow cytometric studies of MCF-7 cells *in vitro* which indicated that $1,25(OH)_2D_3$ and EB1089 increase the percentage of cells in $G_o/ G_1$ and reduce the percentage of cells in S phase (15). Thus, our *in vivo* results with EB1089 correlate well with the *in vitro* reports that vitamin $D_3$ compounds induce both growth arrest and apoptosis in estrogen dependent breast cancer cells (5,14,15,27).

The quantitative data indicate that effect of EB1089 on tumor cell proliferation (two to three fold decrease) was less than the effect of EB1089 on apoptosis (four to eight fold increase). Although actual mean tumor volumes plateau rather than decrease in EB1089 treated mice (Figures 1,2,3), histological examination (Figures 5,6) indicated a reduction in epithelial cells and replacement by stromal tissue in EB1089 treated tumors, support the concept that the epithelial cell compartment has regressed by apoptosis. An effect of EB1089 on tumor cell apoptosis is consistent with our observation that, in two studies, some EB1089 treated tumors regressed completely. Since tumors which underwent complete regression in response to EB1089 were not available for analysis, the apoptotic index in some tumors treated with EB1089 may be even higher than that indicated by the quantitative data presented in Table 1. Our data support the hypothesis that EB1089 has a predominant effect on the apoptotic cell death pathway *in vivo*.

Studies with xenografts derived from MCF-$7^{D3Res}$ cells which display resistance to EB1089 *in vitro* (28), demonstrate that resistance to EB1089 is maintained *in vivo*. Although the basis for vitamin $D_3$ resistance in these cells is unclear, MCF-$7^{D3Res}$ cells (28) and tumors (data not shown) express the vitamin $D_3$ receptor protein at levels comparable to that of MCF-7 cells and tumors. The growth rate of tumors derived from MCF-7 and MCF-$7^{D3Res}$ cells in the absence of treatment was comparable, indicating that tumors selected for vitamin $D_3$ resistance are unlikely to be more aggressive than tumors which are sensitive to vitamin $D_3$. Tumors derived from MCF-$7^{D3Res}$ cells displayed comparable regression in response to estradiol withdrawal, suggesting a functional apoptotic pathway in these tumors which can be activated by other strategies which induce apoptosis. This finding is consistent with our *in vitro* work demonstrating that MCF-$7^{D3Res}$ cells are resistant to EB1089 but sensitive to anti-estrogens such as tamoxifen (28). We are currently examining whether tumors derived from MCF-7 and MCF-$7^{D3Res}$ cells exhibit comparable sensitivity to anti-estrogen induced apoptosis *in vivo* to further test the hypothesis

that anti-estrogens and vitamin $D_3$ compounds act independently to induce apoptosis in breast cancer cells. Support for this hypothesis would suggest that, for patients with mixed tumors containing estrogen dependent and estrogen independent cells, a distinct therapeutic advantage might be achieved by combining agents that activate vitamin $D_3$ mediated apoptosis with those that disrupt estrogen mediated survival signals.

In summary, our studies demonstrate that the vitamin $D_3$ analog EB1089 induces human breast tumor regression by a mechanism which involves both activation of apoptosis and inhibition of proliferation. Our work also indicates that the pathways involved in vitamin $D_3$ mediated apoptosis of MCF-7 tumors are distinct from the pathways which trigger apoptosis in response to estradiol withdrawal. These results support further clinical studies on the therapeutic efficacy of vitamin $D_3$ analogs such as EB1089 against human breast cancer.

## CONCLUSIONS

Our studies demonstrate that the vitamin $D_3$ analog EB1089 induces human breast tumor regression by a mechanism which involves both activation of apoptosis and inhibition of proliferation. Our work also indicates that the pathways involved in vitamin $D_3$ mediated apoptosis of MCF-7 tumors are distinct from the pathways which trigger apoptosis in response to estradiol withdrawal. These studies are designed to address, at the molecular level, the mechanism whereby vitamin $D_3$ analogs and anti-estrogens cooperatively induce breast tumor regression. Our hypothesis predicts that vitamin $D_3$ compounds and anti-estrogens affect distinct sub-populations of breast cells within the tumor, and induce apoptosis via different pathways mediated by the VDR and ER respectively. Support for this hypothesis would imply that vitamin $D_3$ analogs could be clinically useful as adjunctive agents with anti-estrogens, or as a second line treatment for anti-estrogen resistant tumors.

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**STATEMENT OF WORK – REVISED AS OF 5/13/98**

| | |
|---|---|
| **Task 1: months 1-3:** | Comparison of tumor regression induced by the vitamin $D_3$ compound |
| (3 months) | EB1089 and the antiestrogen ICI 182,780 using MCF-7 xenografts in nude mice. |
| Portion complete: | The study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 xenografts in nude mice has been completed. |
| Portion incomplete: | The study of tumor regression induced by the antiestrogen ICI 182,780 using MCF-7 xenografts in nude mice. |
| Revised time: | 1.5 months |
| **Task 2: months 4-9:** | Tumor histopathology, calcium analysis, organ histology, western blotting. |
| (6 months) | |
| Portion complete: | Tumor histopathology, calcium analysis, and organ histology for study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 xenografts in nude mice. |
| Portion incomplete: | Western blotting for study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 xenografts in nude mice. Tumor histopathology, calcium analysis, organ histology, and western blotting for study of tumor regression induced by the antiestrogen ICI 182,780 using MCF-7 xenografts in nude mice. |
| Revised time: | 3 months |
| **Task 3: months 10-12:** | Determination of the effects of EB1089 and ICI 182,780 on growth of tumors |
| (3 months) | derived from vitamin $D_3$ resistant MCF-7 cells. |
| Portion complete: | The study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 $D_3$ resistant xenografts in nude mice has been completed. |
| Portion incomplete: | The study of tumor regression induced by the antiestrogen ICI 182,780 using MCF-7 $D_3$ resistant xenografts in nude mice. |
| Revised time: | 1.5 months |
| **Task 4: months 13-18:** | Tumor histopathology, calcium analysis, organ histology, western blotting |
| (6 months) | |
| Portion complete: | Tumor histopathology, calcium analysis, and organ histology for study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 $D_3$ resistant xenografts in nude mice. |
| Portion incomplete: | Western blotting for study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 $D_3$ resistant xenografts in nude mice. Tumor histopathology, calcium analysis, organ histology, and western blotting for study of tumor regression induced by the antiestrogen ICI 182,780 using MCF-7 $D_3$ resistant xenografts in nude mice. |
| Revised time: | 3 months |
| **Task 5: months 19-21:** | Determination of whether combination treatment with the Vitamin $D_3$ analog |
| (3 months) | and antiestrogen enhances tumor regression compared to either treatment alone. |
| Portion complete: | None |
| Portion incomplete: | All |
| Revised time: | 3 months |
| **Task 6: months 22-27:** | Tumor histopathology, calcium analysis, organ histology, western blotting |
| (6 months) | |
| Portion complete: | None |
| Portion incomplete: | All |
| Revised time: | 6 months |

| | |
|---|---|
| **Task 7: months 28-36:** (9 months) | Final data analysis, manuscript preparation, thesis. |
| Portion complete: | Most data analysis and manuscript preparation for the study of tumor regression induced by the vitamin $D_3$ compound EB1089 using MCF-7 and MCF-7 $D_3$ resistant xenografts in nude mice. |
| Portion incomplete: | All data analysis and manuscript preparation for the study of tumor regression induced by the antiestrogen ICI 182,780 using MCF-7 and MCF-7 $D_3$ resistant xenografts in nude mice.<br>All data analysis and manuscript preparation for the determination of whether combination treatment with the vitamin $D_3$ analog and antiestrogen enhances tumor regression compared to either treatment alone.<br>Thesis. |
| Revised time: | 6 months |

**Total months to completion in original Statement of Work:** **36 months**

**Months elapsed since grant began:** **10.5 months**

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# Apoptotic Regression of MCF-7 Xenografts in Nude Mice Treated with the Vitamin $D_3$ Analog, EB1089*

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**ABSTRACT**

1,25-Dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$] and its synthetic analog EB1089 induce characteristic morphological features of apoptosis in MCF-7 cells *in vitro* that coincide with up-regulation of clusterin and cathepsin B, proteins associated with apoptosis in the mammary gland, and with down-regulation of Bcl-2, an antiapoptotic protein. To determine whether vitamin $D_3$ compounds could mediate apoptosis of breast tumors *in vivo*, we treated nude mice carrying established MCF-7 xenografts with the low calcemic vitamin $D_3$ analog EB1089 via daily injection or sustained release pellets for up to 5 weeks. The volume of tumors from mice treated with 45 pmol/day EB1089 was 4-fold lower than that of tumors from vehicle-treated control mice after 5 weeks. The reduced growth of tumors from EB1089-treated mice was associated with characteristic apoptotic morphology and a marked reduction in the proportion of epithelial cells to stroma. After 5 weeks of treatment with EB1089, MCF-7 tumors exhibited a 6-fold increase in DNA fragmentation (as measured by *in situ* end labeling) relative to that in control tumors. The enhanced rate of apoptosis in tumors from EB1089-treated mice was coupled to a 2-fold reduction in proliferation (as measured by expression of proliferating cell nuclear antigen) compared with that in tumors from control mice. The antitumor effects of EB1089 were evident at doses that had minimal effects on serum calcium and body weight. EB1089 treatment did not alter the growth of xenografts derived from a vitamin $D_3$-resistant variant of MCF-7 cells (MCF-$7^{D3Res}$ cells), which display resistance to EB1089 *in vitro*, indicating that resistance to EB1089 is maintained *in vivo*. Tumors derived from both MCF-7 and MCF-$7^{D3Res}$ cells underwent apoptotic regression upon estradiol withdrawal, indicating comparable estrogen dependence of tumors with differential sensitivity to vitamin $D_3$ compounds. These are the first studies to demonstrate apoptotic morphology and regression of human breast tumors in response to treatment with a vitamin $D_3$ analog *in vivo* and support the concept that vitamin $D_3$ compounds can effectively target human breast cancer. (*Endocrinology* **139:** 2102–2110, 1998)

DESPITE decades of progress in diagnosis, research, and treatment, breast cancer remains the leading cause of cancer death in women. Antiestrogen treatment represents the most effective available endocrine therapy, but is limited to the one third of patients with breast tumors that are estrogen dependent at the time of diagnosis (1). Treatment with antiestrogens in these patients is often followed by the development of a hormone-resistant phenotype (2). Hormone resistance may in part be caused by the selective deletion of hormone-responsive cells by antiestrogen-induced apoptosis in a heterogeneous tumor. It is clear that effective breast cancer therapies must target both estrogen-dependent and -independent cells to minimize the development of hormone-independent tumors with increased metastatic potential.

1,25-Dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$], the biologically active form of vitamin $D_3$ (cholecalciferol), is a potent negative growth regulator of both estrogen-dependent and -independent breast cancer cells *in vitro* (3–7). The vitamin $D_3$ receptor, like the estrogen receptor, is a member of the steroid/thyroid/retinoic acid family of nuclear receptors. Although the estrogen receptor is present in only two thirds of breast tumors, the vitamin $D_3$ receptor is present in over 80% of tumors and does not necessarily colocalize with the estrogen receptor (4, 8). Vitamin $D_3$-based therapeutics thus offer promise as either adjunctive agents for estrogen-dependent tumors or alternative agents for estrogen-independent tumors.

Although it is clear that vitamin $D_3$ compounds inhibit the growth of both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (3–7), the precise mechanism of its effects is unclear. We initially demonstrated that 1,25-$(OH)_2D_3$ induces characteristic features of apoptosis, such as chromatin condensation, nuclear matrix degradation, and DNA fragmentation, in MCF-7 cells *in vitro* (9, 10). Subsequently, we and others reported that apoptosis in breast cancer cells treated with 1,25-$(OH)_2D_3$ or its synthetic analogs is associated with up-regulation of proteins linked to apoptosis in the mammary gland (such as clusterin and cathepsin B) and down-regulation of Bcl-2, an antiapoptotic protein (11–14).

Although 1,25-$(OH)_2D_3$ exerts potent antiproliferative effects *in vivo*, chronic administration induces undesirable hypercalcemic side-effects (4, 15). For this reason, synthetic vitamin $D_3$ compounds have been developed that mimic the antiproliferative effects of 1,25-$(OH)_2D_3$ with less calcemic activity (12, 13, 16–18). Previous studies have demonstrated the efficacy of the synthetic vitamin $D_3$ analog EB1089 (Leo Pharmaceuticals, Ballerup, Denmark) in reducing the growth

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of breast cancer cells and tumors *in vitro* and *in vivo* (12, 17–19). Among several vitamin $D_3$ analogs investigated, EB1089 exhibited the best profile for inhibition of nitrosomethylurea-induced rat mammary tumors in the absence of hypercalcemia (20). The first objective of the studies described here was to determine whether EB1089 could modulate the growth of human breast tumors using a nude mice xenograft model. Our second objective was to determine whether the antitumor effects of EB1089 in human breast tumors involve activation of apoptosis. Our data demonstrate that EB1089 significantly reduces the growth of established human breast tumors by enhancing apoptosis and reducing proliferation of tumor epithelial cells. These data emphasize the potential effectiveness of vitamin $D_3$-based therapeutics for induction of apoptosis in human breast cancer.

## Materials and Methods

### *Cell culture*

MCF-7 human breast cancer cells (obtained from American Type Culture Collection, Rockville, MD) as well as the vitamin $D_3$-resistant variant (MCF-$7^{D3Res}$ cells), which has been previously described (21), were cultured in $\alpha$MEM containing 5% FBS (Life Technologies, Grand Island, NY). Both cell lines were grown in T-150 flasks and yielded 5–10 $\times$ $10^6$ cells/flask depending on confluence. For inoculation into nude mice, cells were washed with PBS, trypsinized, resuspended in $\alpha$MEM, and pooled. After centrifugation, cells were resuspended in Matrigel (Collaborative Biomedical, Waltham, MA)-$\alpha$MEM (4:1).

### *Nude mouse xenograft model*

Three series of studies were conducted to examine the effects of EB1089 on growth and apoptosis of MCF-7 xenografts. In all studies, ovariectomized Ncr *nu/nu* mice (Taconic Farms, Germantown, NY) were implanted sc with 17$\beta$-estradiol-sustained release pellets (Innovative Research, Sarasota, FL). The mice were fed a low calcium (0.1%), purified rodent chow (Purina Test Diets, Richmond, IN) for the duration of the study to minimize the calcemic effects of vitamin $D_3$ analog treatment. Mice were inoculated sc with approximately 5 $\times$ $10^6$ MCF-7 or MCF-$7^{D3Res}$ cells suspended in 0.3 ml Matrigel-$\alpha$MEM. The tumor take rate ranged from 95–100%. Tumor volumes were monitored weekly by caliper measurement of the length, width, and height and were calculated using the formula for a semiellipsoid ($4/3\pi r^3/2$). After 3 weeks, mice bearing tumors with volumes averaging approximately 200 $mm^3$ were randomized for treatment. Because of the variations in tumor take and initial tumor growth as well as the removal of mice for analysis at various time points, the number of mice at each time point varied from experiment to experiment. The number of mice analyzed is reported in the text or figure legends.

In the first series of studies, MCF-7 tumor-bearing mice were treated with EB1089 at a dose of 60 pmol/day. This dose was chosen based on preliminary studies in nontumor-bearing BALB/c mice, which indicated that doses up to 90 pmol/day could be tolerated with little weight loss or elevation of serum calcium when animals were fed the low calcium diet (data not shown). EB1089 was suspended in 80% propylene glycol-20% PBS and administered daily via sc injection. Control mice received daily injections of the vehicle alone. In a third group of tumor-bearing mice, which served as a positive control, estradiol pellets were removed to induce apoptotic tumor regression, as reported by Kyprianou *et al.* (22). Body weights and tumor volumes were monitored weekly, and mice were killed after 2–5 weeks of treatment. At the termination of treatment, mice were anesthetized with sodium pentobarbital, and blood was collected by cardiac puncture for serum calcium determination. Tumors were removed, weighed, and fixed in 4% formalin for histological analysis.

In the second series of studies, the dose of EB1089 was lowered to 45 pmol/day, because mice given 60 pmol EB1089/day experienced weight loss and hypercalcemia. In this experiment, mice bearing MCF-7 tumors were randomized into control (n = 14), EB1089-treated (n = 16), and estradiol withdrawal (n = 3) groups. To determine whether EB1089 induced nonspecific or indirect effects on tumor growth kinetics, tumors were also established from a vitamin $D_3$-resistant variant of MCF-7 cells, termed MCF-$7^{D3Res}$ cells. We have previously demonstrated that MCF-$7^{D3Res}$ cells are resistant to the growth inhibitory effects of EB1089 *in vitro* (21). Mice bearing MCF-$7^{D3Res}$ tumors were randomized into control (n = 8) and EB1089-treated (n = 6) groups in one experiment and into control (n = 3) and estradiol withdrawal (n = 3) groups in another trial. The experimental designs for these studies were otherwise identical to those described for studies employing the 60 pmol/day dose.

To investigate whether EB1089 could be administered via sustained release pellets similar to those used for estrogen supplementation, a preliminary study was conducted with custom-made pellets (Innovative Research) designed to continuously release 30, 45, or 60 pmol EB1089/day for 5 weeks. MCF-7 xenografts were established from MCF-7 cells, and EB1089 or placebo pellets were implanted ip under sodium pentobarbital anesthesia 3 weeks after inoculation. Tumor volumes were measured, and treatment was terminated after 5 weeks. Due to the small numbers of mice in each group (EB1089 pellets, n = 3 for 30 pmol and n = 1 each for 45 or 60 pmol; control, n = 4), the data for all three doses were combined in the analysis.

### *Histological analysis of tumors*

Tumors were embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin (Gill's formulation 3, Fisher Scientific, Fairlawn, NJ) and eosin Y (Sigma Chemical Co., St. Louis, MO). The epithelial nature of the tumors was verified by immunostaining with antibodies directed against epithelia-specific antigen and cytokeratin 18 (data not shown). The mitotic index and apoptotic index were assessed by quantitative morphometric analysis of proliferating cell nuclear antigen (PCNA) expression and *in situ* terminal transferase-mediated fluorescein deoxy-UTP nick end labeling (TUNEL), two established markers of proliferation and apoptosis. For PCNA localization, formalin-fixed, paraffin-embedded sections were incubated for 30 min with a mouse monoclonal anti-PCNA (Nova Castra Laboratories, Newcastle Upon Tyne, UK) at a 1:100 dilution in 1% BSA-PBS. A biotin-conjugated antibody to mouse IgG (Vector Laboratories, Burlingame, CA) was applied at a 1:200 dilution for 30 min in 1% BSA-PBS. The ABC technique was used (avidin and biotinylated horseradish peroxidase complex, Vector) followed by diaminobenzidine (Sigma) to localize peroxidase in the sections, and the sections were counterstained with hematoxylin (Harris modified, Fisher). DNA fragmentation was assessed by TUNEL, using the commercially available assay according to manufacturer's directions (Boehringer Mannheim, Indianapolis, IN). In these sections, nuclei were counterstained with Hoechst 33258 dye (Sigma).

### *Quantitation of apoptosis and proliferation*

PCNA expression and TUNEL were quantitated by viewing and photographing random fields of each tissue section on a Nikon Optiphot-2 microscope and Nikon Microflex UFX-IIA photomicrographic attachment (Nikon Corp., Melville, NY), using a $\times$40 objective. The photographs were scanned and analyzed with the University of Texas Health Science Center at San Antonio Image Tool program, and the percentage of cells staining positively for PCNA or TUNEL was calculated. For both TUNEL and PCNA, 2–6 fields of view (containing at least 250 cells) were quantitated on each section, with 4–8 samples evaluated for each treatment per time point.

### *Statistical analyses*

Statistical comparisons were performed using Student's unpaired $t$ test (for two groups) or one-way ANOVA for more than two groups. Data are expressed as the mean $\pm$ SE, and differences between means were considered significant at $P < 0.05$.

## Results

### *Effect of 60 pmol EB1089 on MCF-7 tumor growth kinetics, body weight, and serum calcium*

Tumor growth kinetics in ovariectomized mice treated with 60 pmol EB1089/day or vehicle in the presence of es-

tradiol supplementation are presented in Fig. 1. In control mice, average tumor volume increased rapidly over the 3-week treatment period. In EB1089-treated mice, tumor volume increased during the first week of treatment and then plateaued, with no further increase in tumor volume between weeks 2–3. In control mice, the mean change in tumor volume between the first and third weeks was 272.3 ± 113.2 $mm^3$ (n = 4) compared with 3.6 ± 15.6 $mm^3$ (n = 5) for tumors from EB1089-treated mice ($P < 0.05$). Tumor volume after 2 weeks was significantly lower in the EB1089 group (351.2 ± 80.4 $mm^3$; n = 10) than in the control group (631.1 ± 65.9 $mm^3$; n = 7). In mice subjected to removal of the estradiol supplementation, tumors regressed rapidly, becoming undetectable within 3 weeks, demonstrating the estrogen dependence of the MCF-7 xenografts.

In contrast to our preliminary data, which indicated that nontumor-bearing nude mice could tolerate doses of EB1089 up to 90 pmol/day, tumor-bearing mice treated with 60 pmol/day EB1089 experienced weight loss and elevated serum calcium levels despite being maintained on the low calcium diet (Table 1).

*Effect of 45 pmol EB1089 on growth of tumors derived from MCF-7 and MCF-7$^{D3Res}$ cells, body weight, and serum calcium*

Although significant effects of EB1089 on MCF-7 tumor growth kinetics were observed in our initial studies, the low tolerance of the tumor-bearing mice to the 60 pmol/day dose precluded definitive conclusions regarding the specificity of this effect. Therefore, a second series of studies using a lower (45 pmol) dose of EB1089 was conducted. In these studies, the effects of EB1089 on tumors derived from a vitamin $D_3$-resistant variant of MCF-7 cells (MCF-7$^{D3Res}$ cells) were also investigated.

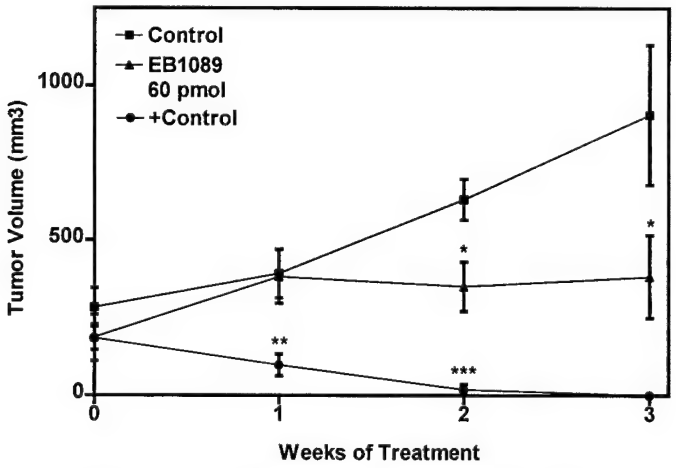


FIG. 1. Effect of 60 pmol/day EB1089 and estradiol withdrawal on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were given daily sc injections of 60 pmol EB1089 or vehicle beginning 3 weeks after tumor cell inoculation. In a separate group of mice, estradiol pellets were removed at time zero, and no further treatment was given. Tumor volumes were monitored weekly and calculated as described in *Materials and Methods*. Each *point* represents the mean ± SE of four control mice, five EB1089-treated mice, and three mice subjected to estradiol withdrawal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

**TABLE 1.** Effect of 60 pmol/day EB1089 on serum calcium and body weight in nude mice bearing MCF-7 tumors

| | Vehicle | EB1089 |
|---|---|---|
| 2 Weeks of treatment | | |
| Serum Ca (mg/dl) | 8.59 ± 0.21 (2) | 12.87 ± 0.05 (2)[a] |
| BW (g) | 21.23 ± 0.84 (4) | 19.08 ± 1.00 (5) |
| 3 Weeks of treatment | | |
| Serum Ca (mg/dl) | 9.41 ± 0.08 (5) | 11.75 ± 1.23 (2)[b] |
| BW (g) | 20.20 ± 0.31 (4) | 16.56 ± 1.23 (5)[a] |

Body weights and serum calcium were measured after 2 or 3 weeks of treatment with 60 pmol/day EB1089 or vehicle. Data are expressed as the mean ± SE, with the number of samples analyzed in *parentheses*.

[a] $P < 0.05$.

[b] $P < 0.01$.

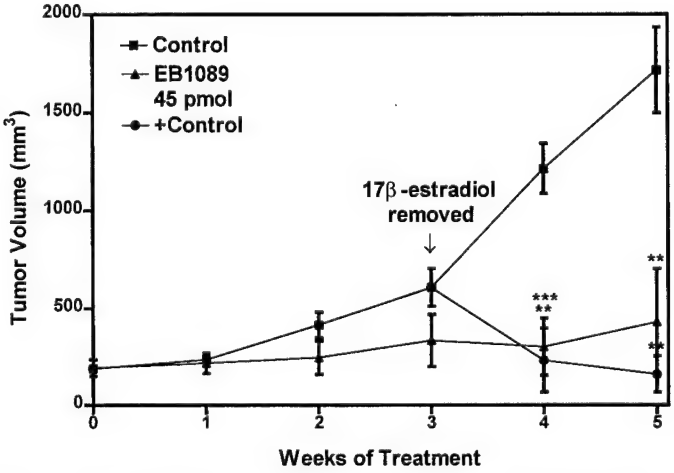


FIG. 2. Effect of 45 pmol/day EB1089 and estradiol withdrawal on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol were inoculated with MCF-7 cells and allowed to grow for 3 weeks. Mice bearing established tumors were then given daily sc injections of 45 pmol EB1089 or vehicle for 5 weeks. In a subset of vehicle-treated mice, estradiol pellets were removed at week 3, and no further treatment was given. In all animals, tumor volumes were monitored weekly. Each *point* represents the mean ± SE of four control mice, five EB1089-treated mice, and three mice subjected to estradiol withdrawal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Similar results were obtained in two independent trials.

Temporal changes in tumor volume for animals bearing MCF-7 tumors and treated with 45 pmol/day EB1089 or vehicle are shown in Fig. 2. This graph shows mean tumor volumes for all animals that completed the 5-week study protocol (*i.e.* not including tumors that were removed for histological analysis at various times). Consistent with the data shown in Fig. 1 for the 60 pmol/day dose, the growth of MCF-7 tumors in mice treated with 45 pmol/day EB1089 was slower than that of tumors in vehicle-treated control animals from 1 week on. The mean change in tumor volume between the first and fifth weeks was 366.6 ± 53.6 $mm^3$ (n = 6) in control mice compared with 53.2 ± 56.9 $mm^3$ (n = 6) in EB1089-treated mice ($P < 0.01$). Tumor volume after 5 weeks was significantly ($P < 0.01$) lower in the EB1089-treated group (428.6 ± 274.0 $mm^3$, n = 6) than in the control group (1716.0 ± 217.7 $mm^3$, n = 6). Final tumor weight was significantly ($P < 0.01$) lower in EB1089-treated mice (0.43 ± 0.27 g; n = 6) than in control mice (1.52 ± 0.19 g; n = 6). These data indicate a good correlation between tumor volume as-

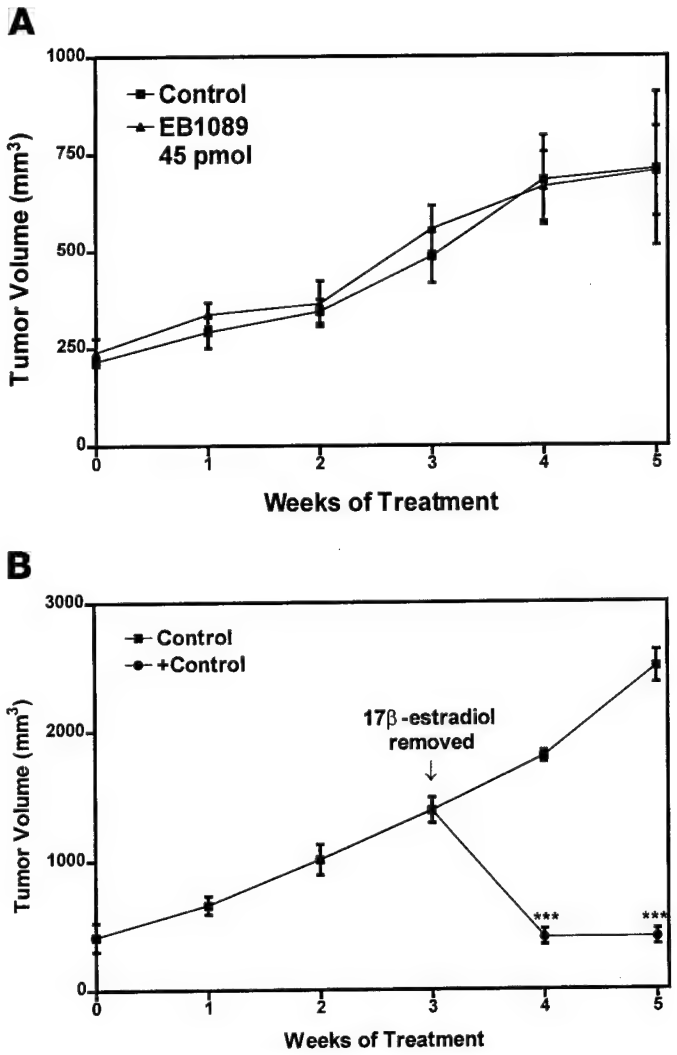


FIG. 3. Effect of 45 pmol/day EB1089 and estradiol withdrawal on growth of MCF-$7^{D3Res}$ tumors. A, Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-$7^{D3Res}$ cells were given daily sc injections of 45 pmol EB1089 or vehicle for 5 weeks. Tumor volumes were monitored as described in Fig. 1. Each *point* represents the mean ± SE of eight control mice and six EB1089-treated mice. Similar results were obtained in three independent treatment trials. B, Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-$7^{D3Res}$ cells were subjected to estradiol withdrawal as described in Fig. 2. In control mice, estradiol pellets were left intact. Tumor volumes were monitored as described in Fig. 1. Each *point* represents the mean ± SE of three control mice and three mice subjected to estradiol withdrawal. ***, Significantly different, control *vs.* estradiol withdrawal, $P < 0.001$. Similar results were obtained in two independent treatment trials.

sessed by caliper measurement in live mice and actual tumor size measured after death. The antitumor effect of EB1089 persisted over the entire 5-week experiment. In addition, although not readily evident from the graph (which shows mean tumor volume), two MCF-7 tumors completely regressed in response to EB1089 treatment. Tumors that regressed were monitored for up to 2 months in the absence of EB1089 treatment, and no regrowth was observed (data not shown). In contrast, spontaneous regression was never observed in tumors from control mice.

**TABLE 2.** Effect of 45 pmol/day EB1089 on serum calcium and body weight of nude mice bearing MCF-7 tumors

| 5 Weeks of treatment | Vehicle | EB1089 |
|---|---|---|
| Serum Ca (mg/dl) | 9.70 ± 0.26 (9) | 10.88 ± 0.67 (6)[a] |
| Corrected BW (g) | 23.68 ± 0.39 (6) | 22.81 ± 0.52 (6) |

Body weights and serum calcium were measured after 5 weeks of treatment with 45 pmol/day EB1089 or vehicle. Because of the significant differences in tumor weights between control and EB1089-treated mice, corrected body weights (total body weight minus tumor weight) are reported. Data are expressed as the mean ± SE, with the number of samples analyzed in *parentheses*.

[a] $P < 0.05$.

In contrast to tumors derived from MCF-7 cells, tumors derived from MCF-$7^{D3Res}$ cells failed to respond to EB1089 treatment (Fig. 3a). There were no significant differences in MCF-$7^{D3Res}$ tumor volumes of control or EB1089-treated mice at any time point throughout the 5-week experiment. As demonstrated in Fig. 3b, MCF-$7^{D3Res}$ tumors underwent rapid regression in response to estradiol withdrawal. Thus, despite resistance to the antitumor effects of EB1089, tumors derived from the MCF-$7^{D3Res}$ cells retained the ability to undergo regression in response to estradiol deprivation, which is known to induce apoptosis in MCF-7 tumors (22).

As indicated in Table 2, although serum calcium was elevated in mice treated for 5 weeks with 45 pmol/day EB1089 compared with that in control mice, the levels were not as high as those in mice receiving 60 pmol/day (Table 1). Furthermore, the significant loss of body weight that was evident with the 60 pmol/day treatment was not observed when EB1089 was given at the 45 pmol/day dose. Corrected final body weight (mouse body weight minus tumor weight) was similar for EB1089-treated and control mice. There were no significant differences in body weight or serum calcium in mice bearing tumors derived from MCF-7 cells compared with those derived from MCF-$7^{D3Res}$ cells regardless of treatment with EB1089 (data not shown). As tumors derived from MCF-$7^{D3Res}$ cells did not respond to EB1089 treatment, the antitumor effect of EB1089 in MCF-7 tumors is probably not due to indirect effects of the vitamin $D_3$ analog on body weight or calcium homeostasis.

### *Effect of EB1089 on morphology, apoptosis, and mitosis of MCF-7 tumors*

Sections from MCF-7 tumors taken from mice treated with 45 or 60 pmol/day EB1089 or vehicle for 2–5 weeks were stained with hematoxylin and eosin for assessment of general morphology. The histological appearances of tumors from mice treated with 45 and 60 pmol/day were similar, and representative micrographs from the 2 week point are presented in Fig. 4. Tumors from vehicle-treated mice were primarily composed of tumor epithelial cells, with small amounts of mouse-derived stroma and frequent blood vessels. The majority of epithelial cells in control tumors were quiescent, although mitotic figures were visible in most sections. Tumors from mice treated with 60 pmol/day EB1089 were composed primarily of quiescent epithelial cells, with few mitotic figures. In many areas, epithelial cells with classic apoptotic morphology (condensed cells with pyknotic nuclei) were frequent. Many EB1089-treated tumors displayed

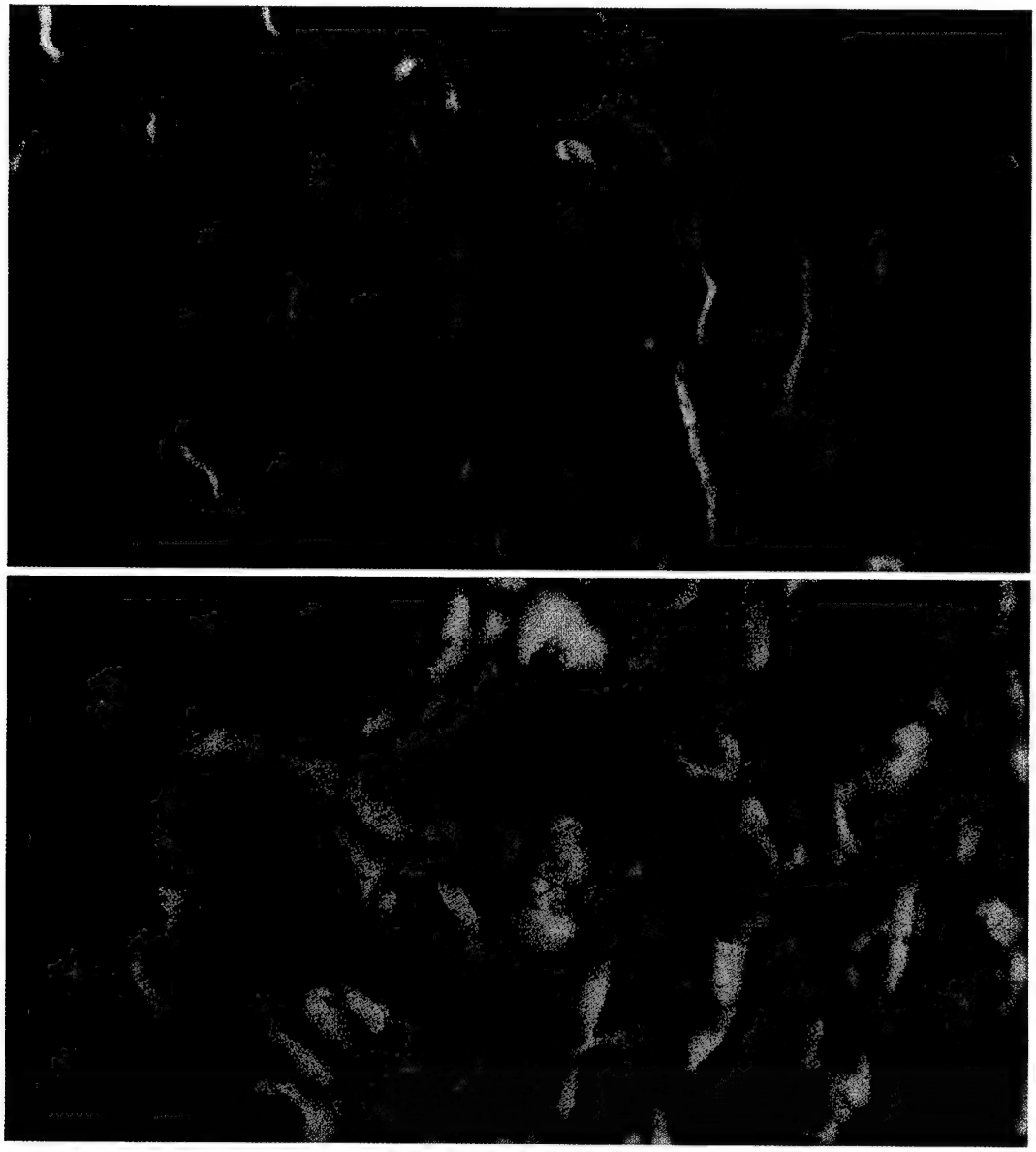

FIG. 4. Effect of EB1089 treatment on MCF-7 tumor morphology. Representative tumor sections from mice treated with vehicle (*top*) or 60 pmol/day EB1089 (*bottom*) for 2 weeks were formalin fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin as described in *Materials and Methods*.

large areas of stroma where deletion of epithelial cells had occurred. The extent of vascularization appeared equivalent in tumors from EB1089-treated and control mice.

To determine whether the changes in MCF-7 tumor morphology induced by EB1089 were associated with changes in the apoptotic or mitotic index, we quantitated the extent of DNA fragmentation (assessed as TUNEL-positive cells) as a marker of apoptosis and expression of PCNA as a marker of proliferation. Representative sections stained with Hoechst dye to visualize nuclear morphology and processed for TUNEL are presented in Fig. 5. Nuclei of epithelial cells in MCF-7 tumors from control mice exhibited normal morphology and were generally negative for DNA fragmentation. In contrast, sections of tumors from EB1089-treated mice exhibited a high prevalence of condensed, irregularly shaped nuclei that were positive for DNA fragmentation. Tumors from mice that had been subjected to estradiol withdrawal also exhibited irregularly shaped, condensed nuclei that were positive for DNA fragmentation. The morphology of tumors from estradiol-deprived mice was similar to that of tumors from EB1089-treated mice, although the extent of apoptosis was higher after estradiol deprivation than after EB1089 treatment (Fig. 5). This finding is consistent with the more pronounced tumor regression in mice subjected to estradiol withdrawal compared with that in mice given EB1089 treatment (Figs. 1–3). Quantitative analysis indicated an increase in the percentage of TUNEL-positive cells in tumors from EB1089-treated mice compared with that in tumors from control mice at all time points examined (Table 3). After 2 weeks of treatment, the higher dose of EB1089 (60 pmol/day) was associated with a more pronounced increase in apoptotic index than the 45 pmol/day dose. After 5 weeks of

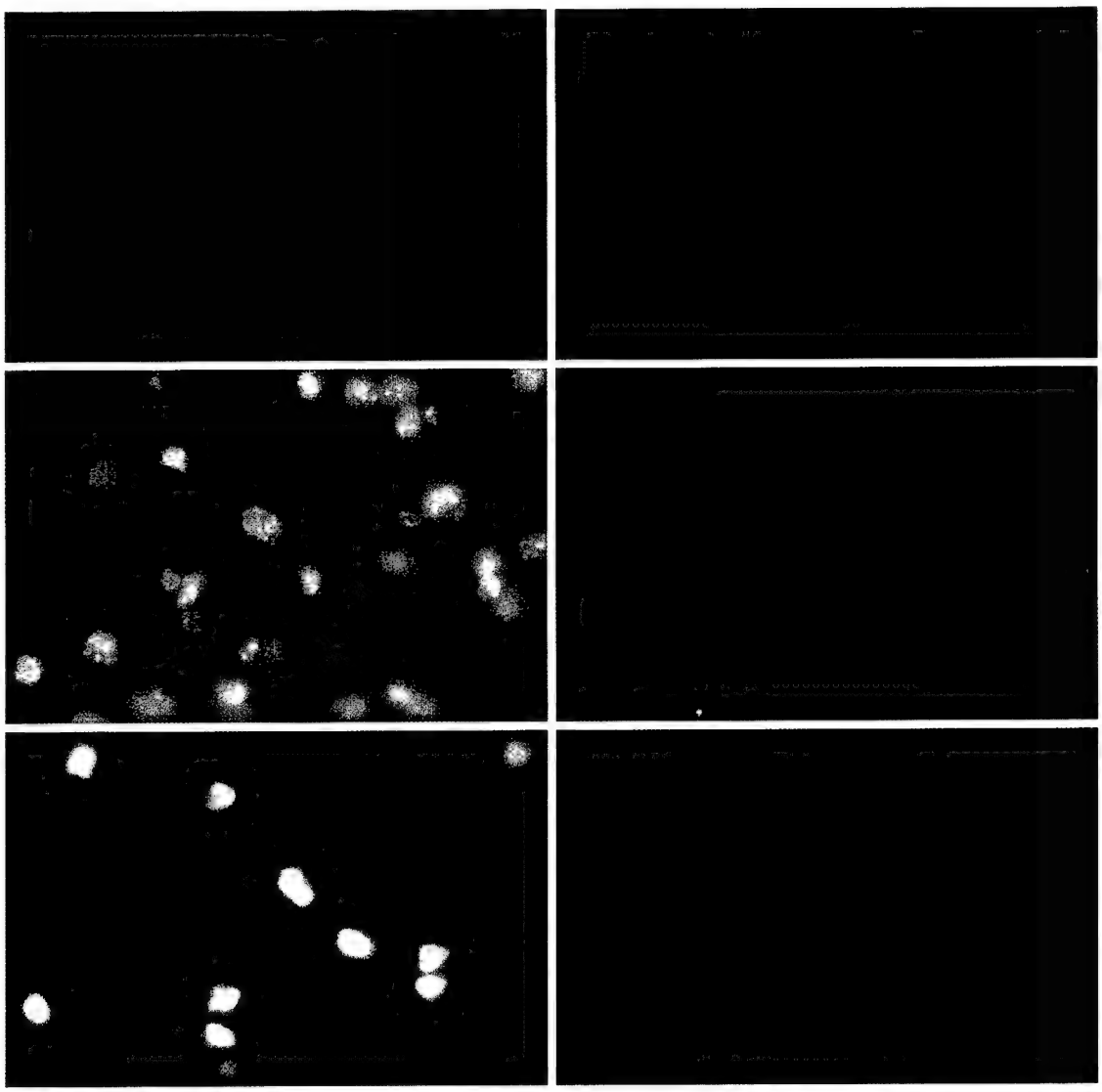

FIG. 5. Effect of EB1089 treatment and estradiol withdrawal on nuclear morphology and DNA fragmentation of MCF-7 tumors. Representative MCF-7 tumor sections from mice treated with vehicle (*top panels*) or 60 pmol/day EB1089 (*middle panels*) or subjected to removal of estradiol supplementation (*bottom panels*) were stained with Hoechst dye to visualize nuclear morphology (*left panels*) and processed for TUNEL (*right panels*) as described in *Materials and Methods*.

treatment, however, both doses of EB1089 were equivalent, with tumors from treated mice exhibiting a 6-fold increase in the percentage of cells positive for DNA fragmentation compared with that in control tumors.

Expression of PCNA was examined as a marker of proliferation in MCF-7 tumor sections derived from control and EB1089-treated mice (Fig. 6). In control tumors, PCNA expression was detected in the nucleus of approximately half of all epithelial cells. A decrease in PCNA expression was detected in tumors from EB1089-treated mice at all time points examined. Quantitation of PCNA expression after 2 weeks indicated a 2.5-fold reduction in PCNA staining in EB1089-treated tumors compared with control tumors (Table 3). Treatments with 45 and 60 pmol/day EB1089 were equally effective in down-regulation of PCNA expression at both time points. The reduced expression of PCNA in EB1089-treated tumors compared with that in control tumors was maintained throughout the 5 weeks of treatment.

*Effect of EB1089 sustained release pellets on growth of MCF-7 tumors*

In a third preliminary study, we examined whether EB1089 could be administered via sustained release pellets similar to those used for estradiol supplementation. Pellets designed to continuously release 30, 45, or 60 pmol EB1089/day or placebo pellets were implanted into mice bearing established MCF-7 tumors. Due to the small number of animals (four control and five EB1089-treated mice) used in this trial, data from the three EB1089 treatment groups were pooled for analysis, as there were no obvious differences in responses to the three doses. As presented in Fig. 7, administration of EB1089 via sustained release pellets elicited a similar antitumor response, as observed in Figs. 1 and 2, for 60 and 45 pmol sc injections. Tumors from mice implanted with EB1089 pellets grew at a slower rate than tumors from mice implanted with placebo pellets from 1 week on. The

**TABLE 3.** Quantitation of PCNA expression and DNA fragmentation in MCF-7 tumors

| | % PCNA | % TUNEL |
|---|---|---|
| 2 Weeks treatment | | |
| Vehicle | 64.52 ± 4.96 | 6.80 ± 2.61 |
| EB1089 (60 pmol/day) | 23.20 ± 9.35[a] | 57.03 ± 3.73[b] |
| Vehicle | 60.11 ± 4.53 | 8.10 ± 1.29 |
| EB1089 (45 pmol/day) | 26.66 ± 6.23[a] | 32.42 ± 2.11[b] |
| 5 Weeks treatment | | |
| Vehicle | 69.99 ± 6.68 | 8.69 ± 2.07 |
| EB1089 (60 pmol/day) | 38.47 ± 1.04[c] | 63.50 ± 3.89[b] |
| Vehicle | 56.20 ± 3.72 | 7.45 ± 0.51 |
| EB1089 (45 pmol/day) | 24.51 ± 7.29[a] | 54.37 ± 3.21[a] |

Tumor sections from mice treated with EB1089 or vehicle were processed for PCNA immunohistochemistry or TUNEL positivity and quantitated as described in *Materials and Methods*. Data are expressed as the mean ± SE of at least 500 cells counted in a minimum of two different fields of view as described in *Materials and Methods*.
[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.

mean change in tumor volume between the first and fifth weeks was 1594.3 ± 418.7 (n = 4) in mice bearing placebo pellets compared with 285.8 ± 164.3 (n = 5) in mice bearing EB1089 pellets ($P < 0.01$), including one EB1089-treated tumor that regressed completely. Final tumor volume at 5 weeks was significantly ($P < 0.05$) lower in the EB1089 pellet group (619.7 ± 256.0 $mm^3$; n = 5) than in the placebo group (2189.6 ± 554.8 $mm^3$; n = 4). There were no significant differences in body weights [control, 24.6 ± 0.2 g (n = 5); EB1089 pellets, 23.6 ± 1.5 g (n = 4)] or serum calcium [control, 9.5 ± 0.2 (mg/dl) (n = 5); EB1089 pellets, 9.6 ± 0.2 (mg/dl) (n = 4)] between mice bearing placebo or EB1089 pellets after 5 weeks.

## Discussion

In this series of *in vivo* studies we have demonstrated that the vitamin $D_3$ analog EB1089 significantly reduces the growth of estrogen-dependent MCF-7 human breast tumors. In our studies, tumor volumes from nude mice treated with EB1089 by daily sc injection at a dose of 45 pmol/mouse·day (~0.8 μg/kg BW) were 4-fold less than those of vehicle-treated mice. At this dose of EB1089, serum calcium was minimally elevated, and no weight loss was observed. These findings complement those of Colston's group (17–20), who demonstrated that oral administration of EB1089 at doses up to 1 μg/kg BW daily slowed the growth of established nitrosomethylurea-induced rat mammary tumors without induction of hypercalcemia. In our nude mice studies, treatment with 60 pmol/mouse·day EB1089 had a more pronounced antitumor effect than treatment with 45 pmol/day, but the higher dose was associated with hypercalcemia, weight loss, and mortality. Preliminary studies using sustained release pellets designed to continuously release EB1089 at doses up to 60 pmol/day indicated that this mode of administration induced antitumor effects similar to those achieved with daily injections. These preliminary data indicated that pellet delivery of EB1089 for 5 weeks was not associated with hypercalcemia or weight loss. As delivery of EB1089 via pellets offers obvious advantages over daily injections (especially when working with nude mice), additional studies to document the actual release rate of EB1089 from pellets and directly assess the efficacy of pellet administration relative to sc injections are warranted.

Histological examination indicated that the decreased size of tumors from EB1089-treated mice, compared with that in control mice, was associated with a reduction in the epithelial component and an increase in the stroma. The 4-fold reduction of tumor volume in mice treated with 45 pmol/day EB1089 for 5 weeks could reflect a decreased rate of cell proliferation, an increased rate of cell death, or both. Our analyses confirmed that EB1089 mediates tumor regression by modulation of both apoptosis and proliferation of tumor epithelial cells.

Quantitative morphometric analysis of DNA fragmentation indicated that tumors from EB1089-treated mice exhibited apoptotic morphology and a 6-fold increase in the percentage of TUNEL-positive cells compared with tumors from control mice. Our studies also demonstrated that MCF-7 tumor regression and DNA fragmentation induced by EB1089 were morphologically similar to tumor regression resulting from estradiol withdrawal, which is known to induce apoptosis in estrogen-dependent MCF-7 tumors (22). Our data demonstrating induction of apoptosis in MCF-7 tumors *in vivo* correlate with earlier findings that demonstrated that 1,25-$(OH)_2D_3$ and its structural analogs induce apoptosis in MCF-7 cells *in vitro* (3, 4, 9–12). In addition to induction of apoptosis, EB1089-treated tumors exhibited a significant decrease in proliferation, as measured by PCNA expression, at all time points examined. These data are consistent with flow cytometric studies of MCF-7 cells *in vitro*, which indicated that 1,25-$(OH)_2D_3$ and EB1089 increase the percentage of cells in $G_0/G_1$ and reduce the percentage of cells in S phase (12). Thus, our *in vivo* results with EB1089 correlate well with the *in vitro* reports that vitamin $D_3$ compounds induce both growth arrest and apoptosis in estrogen-dependent breast cancer cells (5, 11, 12, 14).

The quantitative data indicate that effect of EB1089 on tumor cell proliferation (2- to 3-fold decrease) was less than the effect of EB1089 on apoptosis (4- to 8-fold increase). Although actual mean tumor volumes plateau rather than decrease in EB1089-treated mice, histological examination indicated a reduction in epithelial cells and replacement by stromal tissue in EB1089-treated tumors, supporting the concept that the epithelial cell compartment has regressed by apoptosis. An effect of EB1089 on tumor cell apoptosis is consistent with our observation in two studies that some EB1089-treated tumors regressed completely. As tumors that underwent complete regression in response to EB1089 were not available for analysis, the apoptotic index in some tumors treated with EB1089 may be even higher than that indicated by the quantitative data presented in Table 3. Our data support the hypothesis that EB1089 has a predominant effect on the apoptotic cell death pathway *in vivo*.

Studies with xenografts derived from MCF-$7^{D3Res}$ cells that display resistance to EB1089 *in vitro* (21) demonstrate that resistance to EB1089 is maintained *in vivo*. Although the basis for vitamin $D_3$ resistance in these cells is unclear, MCF-$7^{D3Res}$ cells (20) and tumors (data not shown) express the vitamin $D_3$ receptor protein at levels comparable to those in

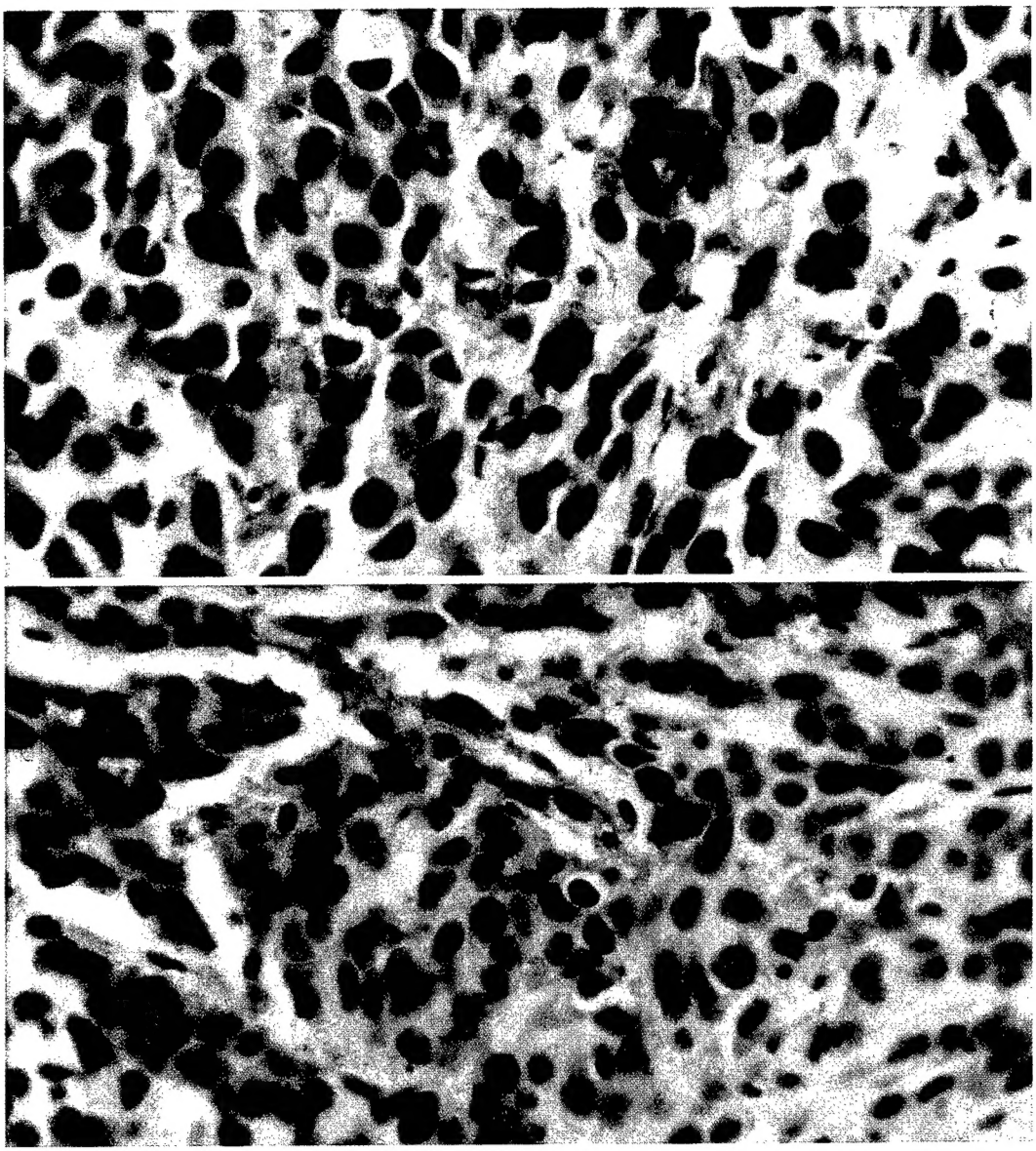

FIG. 6. Effect of EB1089 treatment on PCNA expression in MCF-7 tumors. Expression of PCNA in representative tumor sections from mice treated with vehicle (*top*) or 60 pmol/day EB1089 (*bottom*) for 2 weeks. Sections were incubated with a mouse monoclonal antibody directed against human PCNA and developed with the ABC technique as described in *Materials and Methods*. PCNA expression appears as brown nuclear staining.

MCF-7 cells and tumors. The growth rates of tumors derived from MCF-7 and MCF-$7^{D3Res}$ cells in the absence of treatment were comparable, indicating that tumors selected for vitamin $D_3$ resistance are unlikely to be more aggressive than tumors that are sensitive to vitamin $D_3$. Tumors derived from MCF-$7^{D3Res}$ cells displayed comparable regression in response to estradiol withdrawal, suggesting a functional apoptotic pathway in these tumors that can be activated by other strategies that induce apoptosis. This finding is consistent with our *in vitro* work demonstrating that MCF-$7^{D3Res}$ cells are resistant to EB1089 but sensitive to antiestrogens such as tamoxifen (21). We are currently examining whether tumors derived from MCF-7 and MCF-$7^{D3Res}$ cells exhibit comparable sensitivity to antiestrogen-induced apoptosis *in vivo* to further test the hypothesis that antiestrogens and vitamin $D_3$ compounds act independently to induce apoptosis in breast cancer cells. Support for this hypothesis would suggest that for patients with mixed tumors containing estrogen-dependent and estrogen-independent cells, a distinct therapeutic advantage might be achieved by combining agents that activate vitamin $D_3$-mediated apoptosis with those that disrupt estrogen-mediated survival signals.

In summary, our studies demonstrate that the vitamin $D_3$ analog EB1089 induces human breast tumor regression by a mechanism that involves both activation of apoptosis and inhibition of proliferation. Our work also indicates that the pathways involved in vitamin $D_3$-mediated apoptosis of MCF-7 tumors are distinct from the pathways that trigger apoptosis in response to estradiol withdrawal. These results

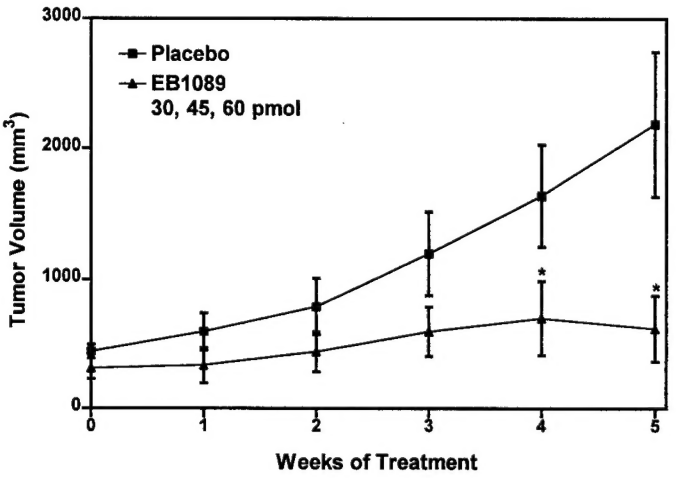


FIG. 7. Effect of EB1089 sustained release pellets on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were implanted ip with sustained release EB1089 pellets, and tumor volumes were monitored for 5 weeks. Data are expressed as the mean ± SE of four control and five EB1089-treated mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

support further clinical studies on the therapeutic efficacy of vitamin $D_3$ analogs such as EB1089 against human breast cancer.

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